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THÈSE présentée par :

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Analyse mécanistique des traitements de la douleur neuropathique

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"La plus grande gloire n'est pas de ne jamais tomber, mais de se relever à chaque chute."

Confucius

SOMMAIRE

LISTE DES ABRÉVIATIONS
(contenues dans les parties en français)

α_2-AR	Récepteur α_2-adrénergique
β_2-AR	Récepteur β_2-adrénergique
BDNF	Brain-Derived Neurotrophic Factor (*facteur neurotrophique dérivé du cerveau)*
CO	Monoxyde de carbone
DOP	Delta des opioïdes
ELISA	Enzyme-linked immunosorbent assay (*dosage d'immunoabsorption par enzyme liée*)
GABA	Acide γ-aminobutyrique
GFAP	Glial Fibrillary Acidic Protein (*protéine acide fibrillaire gliale*)
HO-1	Hème oxygénase 1
IASP	International Association for the Study of Pain
IL	Interleukine
KOP	Kappa des opioïdes
MCP-1	Monocyte Chemoattractant Protein-1 (*protéine 1 chimioattractive monocytaire)*
MMP	Métalloprotéase
MOP	Mu des opioïdes
mTNFα	Forme membranaire du facteur de nécrose tumorale α
NFκB	Nuclear Factor kappa B (*facteur de transcription nucléaire kappa B*)
NGF	Nerve Growth Factor (*facteur de croissance neural*)
NNT	Number-Needed-to-Treat (*nombre de sujets à traiter*)
nor-BNI	Norbinaltorphimine
RT-PCR	Real-Time Polymerase Chain Reaction (*réaction en chaîne polymérase en temps réel*)
SSNRI	Inhibiteur sélectif de la recapture de la sérotonine et de la noradrénaline
TACE	Enzyme de conversion du facteur de nécrose tumorale α
TH	Tyrosine Hydroxylase
TNFα	Facteur de nécrose tumorale α
TNFR1	Récepteur 1 du facteur de nécrose tumorale
TNFR2	Récepteur 2 du facteur de nécrose tumorale

PUBLICATIONS ET COMMUNICATIONS

PUBLICATIONS ISSUES DES TRAVAUX PRÉCURSEURS :

Antidepressants suppress neuropathic pain by a peripheral β_2-adrenoceptor mediated anti-TNFα mechanism.
Bohren Y, Tessier LH, Megat S, Petitjean H, Hugel S, Daniel D, **Kremer M**, Fournel S, Hein L, Schlichter R, Freund-Mercier MJ, Yalcin I, Barrot M. ***Neurobiol Dis***, 2013, 60:39-50.

The sciatic nerve cuffing model of neuropathic pain in mice.
Yalcin I, Megat S, Barthas F, Waltisperger E, **Kremer M**, Salvat E, Barrot M. ***J Vis Exp***, 2014, 89:e51608.

PUBLICATIONS DE THÈSE :

The antiallodynic action of pregabalin in neuropathic pain is independent from the opioid system.
Kremer M, Nexon L, Wurtz X, Ceredig RA, Daniel D, Hawkes R, Yalcin I, Salvat E, Barrot M. ***Mol Pain,*** 2016, 12:1744806916636387.

Antidepressants and gabapentinoids in neuropathic pain: mechanistic insights.
Kremer M, Salvat E, Muller A, Yalcin I, Barrot M. ***Neuroscience***, 2016, (*sous presse*).

A dual mechanism for duloxetine relief of neuropathic allodynia.
Kremer M, Yalcin I, Goumon Y, Ceredig RA, Nexon L, Daniel D, Megat S, Chavant V, Lacaud A, Lelievre V, Gilsbach R, Lutz PE, Salvat E, Barrot M. *(en écriture).*

δ opioid receptors are essential to the antiallodynic action of β_2-mimetics.
Kremer M, Wurtz X, Megat S, Bohren Y, Nexon L, Ceredig RA, Daniel D, Yalcin I, Barrot M. *(en écriture).*

COMMUNICATIONS AFFICHÉES :

Anti-TNFα drug relief of neuropathic pain in mice.
Kremer M, Megat S, Daniel D, Freund-Mercier MJ, Yalcin I, Barrot M.
Meeting annuel Neurex, Strasbourg, France, Octobre 2012.

Antidepressants drugs relieve neuropathic allodynia by a peripheral β_2-adrenoceptor mediated anti-TNFα mechanism.
Yalcin I, Bohren Y, Tessier LH, Megat S, Petitjean H, Hugel S, **Kremer M**, Daniel D, Hein L, Schlichter R, Feund-Mercier MJ, Barrot M.
4th International Congress on Neuropathic Pain, Toronto, Canada, Mai 2013.

Antidepressants drugs relieve neuropathic allodynia by a peripheral β_2-adrenoceptor mediated anti-TNFα mechanism.
Freund-Mercier MJ, Bohren Y, Tessier LH, Petitjean H, Megat S, **Kremer M**, Daniel D, Hein L, Schlichter R, Hugel S, Yalcin I, Barrot M.
Société des Neurosciences, 11ème Colloque, Lyon, France, Mai 2013.

Mechanism of the antiallodynic effect of chronic oral duloxetine in mice with sciatic nerve cuffing.
Kremer M, Yalcin I, Wurtz X, Ceredig RA, Daniel D, Megat S, Hawkes R, Salvat E, Barrot M.
5th International Congress on Neuropathic Pain, Nice, France, Mai 2015.

COMMUNICATION ORALE :

Dualité d'action de la duloxétine dans un modèle murin de douleur neuropathique.
Kremer M, Yalcin I, Goumon Y, Ceredig RA, Nexon L, Daniel D, Megat S, Chavant V, Lacaud A, Lelievre V, Gilsbach R, Lutz PE, Salvat E, Barrot M.
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AVANT-PROPOS

La douleur neuropathique est liée à une lésion ou une pathologie du système nerveux et plus précisément du système somatosensoriel (Loeser and Treede, 2008). Une étude menée en France estime autour de 6% la prévalence de douleurs chroniques avec composante neuropathique (Bouhassira et al., 2008). En plus de la souffrance physique qu'elle génère, la douleur neuropathique altère la qualité de vie au quotidien, causant une souffrance, une irritabilité, parfois une agressivité, un repli sur soi, voire une perte d'autonomie et augmente le risque de développer des troubles de l'humeur. Elle a aussi un coût socio-économique important par ses répercussions sur la vie professionnelle. La prise en charge de la douleur neuropathique est donc un défi de santé publique.

Certains antidépresseurs et anticonvulsivants sont actuellement les meilleures thérapies disponibles (Finnerup et al., 2015). Leur efficacité a été découverte de façon fortuite par les cliniciens, puis confirmée par des études contrôlées. Cependant leur prescription n'est pas efficace chez tous les patients et les effets indésirables conduisent 10 à 30 % d'entre eux à arrêter le traitement. Comprendre le mécanisme d'action de ces traitements sur la douleur neuropathique périphérique peut être important pour améliorer leur efficacité, cibler les effets bénéfiques et limiter les effets indésirables.

Personnellement concernée par les douleurs neuropathiques, je me suis présentée en 2011 auprès de l'équipe du Dr. Michel Barrot pour faire un stage de Master 2 sur ce sujet et poursuivre ces travaux en thèse. En effet, depuis la caractérisation comportementale d'un modèle de douleur neuropathique périphérique chez la souris en 2008, cette équipe a réalisé plusieurs études proposant un mécanisme d'action des antidépresseurs lors d'un traitement prolongé. Grâce à ce modèle, il a été montré que ces molécules agissent sur l'allodynie neuropathique, une douleur déclenchée par un stimulus non nociceptif, en recrutant la noradrénaline qui va agir via les récepteurs β_2-adrénergiques (β_2-AR) (Yalcin et al., 2009a; Yalcin et al., 2009b). Ce mécanisme d'action est également dépendant des récepteurs delta des opioïdes (DOP) (Benbouzid et al., 2008b; Bohren et al., 2010; Megat et al., 2015). Suite à ces travaux, il a été proposé chez l'animal que des agonistes β_2-adrénergiques seraient bénéfiques face à la douleur neuropathique périphérique (Choucair-Jaafar et al., 2009; Yalcin et al., 2010) ; hypothèse supportée ensuite en clinique où un report de cas suggère un effet des β_2-mimétiques dans le soulagement de la douleur neuropathique chez 6 patients (Cok et al., 2010) et où une étude épidémiologique rétrospective récente

rapporte une diminution de l'incidence des douleurs neuropathiques post-thoracotomie chez des patients sous traitement au long cours par des β_2-mimétiques (Salvat et al., 2015).

De nombreuses zones d'ombre restaient toutefois présentes dans la compréhension du mécanisme par lequel les antidépresseurs, et potentiellement les β_2-mimétiques, soulagent la douleur neuropathique lorsque j'ai commencé mon stage en 2012.

Le premier chapitre de cette thèse présente les travaux en cours à mon arrivée en stage. Ces travaux auxquels j'ai contribué, publiés depuis, ont servi de point de départ à mes travaux de thèse.

Le premier article concerne le modèle murin de douleur neuropathique périphérique caractérisé par l'équipe ; modèle que j'ai utilisé pour l'ensemble de mes travaux de thèse :

- The sciatic nerve cuffing model of neuropathic pain in mice. Yalcin I, Megat S, Barthas F, Waltisperger E, Kremer M, Salvat E, Barrot M. *J Vis Exp*, 2014, 89:e51608.

Le second article traite de la localisation du mécanisme d'action thérapeutique de la nortriptyline, un antidépresseur tricyclique, ainsi que de son action neuroimmunitaire en particulier sur le facteur de nécrose tumorale α (TNFα). Cet antidépresseur, ainsi que la venlafaxine, un inhibiteur sélectif de la recapture de la sérotonine et de la noradrénaline (SSNRI), et la terbutaline, un β_2-mimétique, présentent en effet une action anti-TNFα dans les ganglions rachidiens lors d'une compression de nerf sciatique. Mes travaux de master ont permis de compléter ces données en testant des molécules anti-TNFα, utilisées en clinique dans des pathologies inflammatoires, sur l'allodynie mécanique dans ce modèle murin.

- Antidepressants suppress neuropathic pain by a peripheral β_2-adrenoceptor mediated anti-TNFα mechanism. Bohren Y, Tessier LH, Megat S, Petitjean H, Hugel S, Daniel D, Kremer M, Fournel S, Hein L, Schlichter R, Freund-Mercier MJ, Yalcin I, Barrot M. *Neurobiol Dis*, 2013, 60:39-50.

Le deuxième chapitre, présenté sous forme de revue de la littérature, synthétise l'état des connaissances actuelles sur les mécanismes d'action des antidépresseurs et des gabapentinoïdes dans divers modèles murins :

- Antidepressants and gabapentinoids in neuropathic pain: mechanistic insights. Kremer M, Salvat E, Muller A, Yalcin I, Barrot M. *Neuroscience*, 2016, (*sous presse*).

Le troisième chapitre présente mes travaux originaux de thèse.

La première partie concerne l'implication du système opioïdergique dans l'action antiallodynique de la prégabaline et fait l'objet d'une publication :

- The antiallodynic action of pregabalin in neuropathic pain is independent from the opioid system. Kremer M, Nexon L, Wurtz X, Ceredig RA, Daniel D, Hawkes R, Yalcin I, Salvat E, Barrot M. *Mol Pain,* 2016, 12:1744806916636387.

La deuxième partie explore le mécanisme d'action de la duloxétine. Cette étude translationnelle est actuellement en cours d'écriture :

- A dual mechanism for duloxetine relief of neuropathic allodynia. Kremer M, Yalcin I, Goumon Y, Ceredig RA, Nexon L, Daniel D, Megat S, Chavant V, Lacaud A, Lelievre V, Gilsbach R, Lutz PE, Salvat E, Barrot M. *(en écriture).*

La dernière partie a pour objectif d'évaluer le rôle du système opioïdergique dans l'effet antiallodynique des β_2-mimétiques. Ce travail fait l'objet d'un article actuellement en cours d'écriture :

- δ opioid receptors are essential to the antiallodynic action of β-mimetics. Kremer M, Wurtz X, Megat S, Bohren Y, Nexon L, Ceredig RA, Daniel D, Yalcin I, Barrot M. *(en écriture).*

Une discussion générale viendra critiquer et discuter ces données pour essayer d'établir des hypothèses originales sur les traitements de la douleur neuropathique.

TRAVAUX PRÉCURSEURS

I. The sciatic nerve cuffing model of neuropathic pain in mice.

Yalcin I, Megat S, Barthas F, Waltisperger E, **Kremer M**, Salvat E, Barrot M. ***J Vis Exp***, 2014, 89:e51608. http://www.jove.com/video/51608/the-sciatic-nerve-cuffing-model-of-neuropathic-pain-in-mice

De part sa chronicité, sa résistance aux antalgiques usuels et ses conséquences sur la vie quotidienne, la prise en charge de la douleur neuropathique est un enjeu majeur. Afin de progresser dans la connaissance des mécanismes physiopathologiques de ce type de douleur et d'en améliorer la prise en charge thérapeutique, la recherche préclinique a besoin de modèles animaux reproduisant cette douleur et sa réponse aux traitements existants. Dans ce contexte, divers modèles ont été caractérisés, principalement chez le rongeur. Ces modèles ont pour objectif de reproduire une symptomatologie neuropathique douloureuse soit par une lésion nerveuse (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; Decosterd and Woolf, 2000), soit en mimant une maladie connue pour affecter le système somatosensoriel, telle que le diabète (Allen et al., 2004; Choucair-Jaafar et al., 2014).

Malgré le nombre important de modèles animaux développés sur le sujet, la majorité des études réalisées dans un contexte de douleur neuropathique utilise des modèles de lésion mécanique du système nerveux périphérique et en particulier du nerf sciatique (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; Decosterd and Woolf, 2000). Dans le but de limiter la variabilité inter-expérimentateur liée aux techniques de lésion, Mosconi et Kruger ont décrit un modèle dans lequel un manchon en polyéthylène de taille standardisée est placé de façon unilatérale autour du tronc commun du nerf sciatique chez le rat : le modèle dit du « cuff » (Mosconi and Kruger, 1996). Ce modèle présente certains avantages : il est facilement reproductible d'un expérimentateur à l'autre mais également d'un animal à l'autre ; il reproduit la sensibilité de la douleur neuropathique aux traitements existants tels que les antidépresseurs et les anticonvulsivants ; le nerf sciatique est un nerf facile d'accès ce qui permet une chirurgie rapide dans le respect du bien-être animal ; enfin le nerf sciatique innerve un vaste territoire cutané permettant de réaliser facilement des tests nociceptifs sur les pattes postérieures de l'animal.

Dans ce modèle, la douleur neuropathique est induite par la compression du tronc commun du nerf sciatique. La chirurgie est effectuée sous anesthésie générale. Après avoir mis en évidence puis dégagé le nerf sciatique droit, un manchon de polyéthylène long de

2 mm, de diamètre interne de 0,38 mm et de diamètre externe de 1,09 mm, est placé autour du tronc commun. La peau est ensuite suturée et une allodynie mécanique persistante, un des symptômes de la douleur neuropathique, apparait les jours suivant la pose du manchon.

Ce modèle n'est pas le plus utilisé dans la littérature scientifique, mais il a conduit à des résultats importants. Il a ainsi permis la mise en évidence d'un mécanisme de signalisation glie/neurone, au sein de la moelle épinière, qui sous-tend les douleurs neuropathiques (Coull et al., 2003; Coull et al., 2005; Zhang and De Koninck, 2006; Keller et al., 2007). Il a également été utilisé pour étudier les changements morphologiques et fonctionnels des fibres afférentes primaires (Pitcher and Henry, 2000, 2004; Thakor et al., 2009; Zhu and Henry, 2012; Zhu et al., 2012), ainsi que l'implication des différents récepteurs des opioïdes dans le soulagement de la douleur neuropathique par des agonistes opioïdergiques mais également par des antidépresseurs (Holdridge and Cahill, 2007; Kabli and Cahill, 2007; Benbouzid et al., 2008a; Benbouzid et al., 2008b). Enfin, ce modèle est également approprié pour étudier le mécanisme d'action des antidépresseurs ainsi que les conséquences émotionnelles de la douleur neuropathique (Benbouzid et al., 2008a; Benbouzid et al., 2008b; Choucair-Jaafar et al., 2009; Yalcin et al., 2009a; Yalcin et al., 2009b; Bohren et al., 2010; Yalcin et al., 2010; Yalcin et al., 2011; Bohren et al., 2013; Barthas et al., 2015; Megat et al., 2015).

La technique chirurgicale de ce modèle du « cuff », ainsi que la procédure du test nociceptif des filaments de von Frey permettant de mesurer la présence d'une allodynie mécanique statique, ont été filmées afin de faciliter la diffusion et l'utilisation de ce modèle murin de neuropathie périphérique au sein d'autres équipes de recherche. Dans cet article vidéo publié dans *The Journal of Visualized Experiments* (JoVE), la chirurgie a été réalisée par le Dr. Ipek Yalcin et les tests nociceptifs par le Dr. Salim Megat. L'ensemble des doctorants de l'équipe ont participé à la préparation de cet article, ils ont notamment effectué une recherche bibliographique complète afin d'obtenir l'ensemble des publications utilisant ce modèle. Cette recherche de la littérature a été réalisée en utilisant différentes bases de données scientifiques, telles que Pubmed et Scopus. J'ai personnellement participé à ce travail bibliographique en effectuant une recherche inversée sur Scopus, afin d'obtenir l'ensemble des publications citant les articles princeps (Mosconi and Kruger, 1996; Fisher et al., 1998; Pitcher et al., 1999), ainsi que celles citant les travaux de notre équipe.

Video Article

The Sciatic Nerve Cuffing Model of Neuropathic Pain in Mice

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Abstract

Neuropathic pain arises as a consequence of a lesion or a disease affecting the somatosensory system. This syndrome results from maladaptive changes in injured sensory neurons and along the entire nociceptive pathway within the central nervous system. It is usually chronic and challenging to treat. In order to study neuropathic pain and its treatments, different models have been developed in rodents. These models derive from known etiologies, thus reproducing peripheral nerve injuries, central injuries, and metabolic-, infectious- or chemotherapy-related neuropathies. Murine models of peripheral nerve injury often target the sciatic nerve which is easy to access and allows nociceptive tests on the hind paw. These models rely on a compression and/or a section. Here, the detailed surgery procedure for the "cuff model" of neuropathic pain in mice is described. In this model, a cuff of PE-20 polyethylene tubing of standardized length (2 mm) is unilaterally implanted around the main branch of the sciatic nerve. It induces a long-lasting mechanical allodynia, *i.e.*, a nociceptive response to a normally non-nociceptive stimulus that can be evaluated by using von Frey filaments. Besides the detailed surgery and testing procedures, the interest of this model for the study of neuropathic pain mechanism, for the study of neuropathic pain sensory and anxiodepressive aspects, and for the study of neuropathic pain treatments are also discussed.

Video Link

The video component of this article can be found at http://www.jove.com/video/51608/

Introduction

Neuropathic pain is usually chronic and arises as a consequence of a lesion or a disease affecting the somatosensory system. Maladaptive changes in injured sensory neurons and along the entire nociceptive pathway within the central nervous system participate in this complex syndrome. Various models have been developed in rodents for studying neuropathic pain and its treatments[1-3].

Based on known etiologies, the models of neuropathic pain aim at mimicking the polyneuropathy observed in diabetes, the injuries to peripheral nerves, the central injuries, the trigeminal neuralgia, the neuropathies consecutive to chemotherapy, the post-herpetic neuralgia, etc. Different models of peripheral nerve injury in rodents focus on the sciatic nerve. These models depend on a compression and/or a section of this nerve. Indeed, the sciatic nerve affords relative easy surgery and allows for tests based on paw withdrawal reflexes. The models of chronic nerve compression include for example: the chronic constriction injury (CCI)[4,5], the sciatic nerve cuffing[6-9], the partial sciatic nerve ligation (PSL)[10], the spinal nerve ligation (SNL)[11], or the common peroneal nerve ligation[12]. Models referred to as "spared nerve injury" (SNI) are also widely used. They consist of a tight ligation and axotomy of two out of the three terminal branches of the sciatic nerve, while the third branch remains intact[13-15]. The various models of neuropathic pain, which target the sciatic nerve, result in a chronic mechanical allodynia (a nociceptive response to a normally non-nociceptive stimulus) on the injured hind paw.

Here, the detailed surgery procedure for the "cuff model" of neuropathic pain in mice is described. It consists in the implantation of a polyethylene cuff around the main branch of the sciatic nerve[6-9]. The use of von Frey filaments is also described. These filaments allow assessing the mechanical allodynia which is a long lasting nociceptive symptom present in this model.

Protocol

Protocols have been approved by the "comité d'éthique en matière d'expérimentation animale de Strasbourg" (CREMEAS).

1. Baseline Measurement of Paw Withdrawal Thresholds

1. Allow the mice to habituate to the animal facilities for at least 10 days to 2 weeks before initiating the testing procedures.
2. Habituate the mice to the von Frey testing set-up and to the von Frey procedure that are described in section 4.

3. Before surgery, evaluate the mechanical paw withdrawal thresholds with von Frey filaments as described in the section 4.3. Note: Repeat the procedure on separate days until at least three stable consecutive values are obtained for paw withdrawal thresholds.
4. Assign the mice to the different experimental groups so that these groups do not initially differ for paw withdrawal thresholds.

2. Surgery Procedure for Cuff Implantation

1. Weigh the animal. Note: Mouse body weight should be over 20 g for the cuff insertion procedure described below.
2. Anesthetize the animal with an intraperitoneal injection of 4 ml/kg of a mixture of ketamine (17 mg/ml) and xylazine (2.5 mg/ml) in 0.9% NaCl, which provides around 45 min of anesthesia.
3. Check the absence of paw reflexes by pinching a hind paw with tweezers and check the absence of eye reflexes to make sure that the animal is fully anesthetized.
4. Shave the right leg from the knee to the hip using an electrical shaver.
5. Apply protective eye liquid gel to the eyes with a cotton-tipped swab.
6. Place the animal on its left side and place the right hind limb on a small pillow and maintain the right hind limb to the pillow with adhesive tape.
7. Disinfect the surgery field with chlorhexidine and 70% ethanol using gauze pad or cotton-tipped swab.
8. Find the femur using the forefinger and make an incision of approximately 0.5 cm, parallel to the femur and approximately 1.5 mm anterior to the femur.
9. Separate the muscles close to the femur with two autoclaved sticks. Notes: Never cut the muscle. Normally, the muscle layers separate easily without any bleeding and the sciatic nerve is then visible. In case of bleeding, use a sterile cotton-tipped swab to absorb the blood.
10. Insert two autoclaved sticks below the sciatic nerve to expose its main branch, and hydrate the nerve with a sterile physiological solution (0.9% NaCl).
11. Hold the pre-prepared sterile 2 mm section of split PE-20 polyethylene tubing (cuff), 0.38 mm ID / 1.09 mm OD, with the help of a pointed steel stick and a bulldog clamp.
    1. Insert the pointed steel stick into the cuff, which will slightly open it.
    2. Using the cuff lateral opening, insert the bulldog at one end of the cuff and parallel to the cuff. Rotate the bulldog (180°) so that it will hold the cuff by the side that is opposite to the lateral opening. Close the bulldog and remove the pointed steel stick. Note: The rotation is done to allow holding-on the cuff in an optimized position for the insertion, the bulldog clamp is also helping to maintain the cuff partly open. The model and the size of the bulldog clamp are critical for this step of the procedure.
12. Have a second experimenter hold the two sticks under the nerve and gently separate the sticks to facilitate the access to a section of sciatic nerve that is around 4 mm long.
13. Insert the 2 mm cuff around the main branch of the sciatic nerve, starting by inserting the part of the cuff that is distal to the bulldog around the part of the nerve that is proximal to the hip.
14. Close the cuff gently by exerting pressure on its two distal sides with pliers, without squeezing or changing the form of the tube. Turn the cuff to ensure that it is closed correctly.
15. Suture the shaved skin layer with surgical knots.
16. Place the mouse on its left side in a clean home cage. Keep it under the heat lamp until the mouse is awake.
17. Add extra water and place some chow directly in the home cage.

3. Surgery Procedure for Sham Controls

1. Apply the same surgery procedure as described above from step 2.1 to step 2.9, then follow with steps 2.15 to 2.17. For sham controls, omit the steps 2.10 to 2.14 that only concern the cuff insertion.

4. von Frey Testing

1. Place the mice in clear individual boxes (7 cm x 9 cm x 7 cm) with holes, on an elevated perforated plate of smooth stainless steel (1 m x 50 cm, 5 mm circle perforations with 2.5 mm between perforation borders). Note: Up to 12 mice can be concomitantly tested on this set-up. Operated animals can be tested the day after the surgery. However, 3 days of recovery are recommended to diminish the post-surgery hypersensitivity observed in sham controls.
2. Allow the animals to habituate for 15 min prior to testing.
3. Apply the von Frey filaments to the plantar surface of each hind paw in a series of ascending forces. Notes: The von Frey filaments are plastic hairs of calibrated diameters. They are 5 cm long and are fixed on hand-held applicators. The speed of filament application, the degree of bending and the duration of the application can influence the threshold values that are obtained with this test[3]. With the present procedure in mice, the filaments that are the most often used are the 0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, and 10 g.
    1. Apply the chosen filament to the plantar surface of the left paw until the filament just bends. Repeat the procedure three to five consecutive times, and then do the same to the right paw. Once the filament has been tested on both paws, test the next animal. Notes: Avoid paw lateral borders which can be more sensitive. The expected response is a paw withdrawal, sudden flinching or paw licking. Consider the response as positive if at least three expected responses are observed out of five trials. A given paw is always tested three times, but the fourth and the fifth trials are done only if 1 or 2 response(s) was (were) observed during the first three tests. In C57BL/6J mice, start the pre-surgery tests with the 1.4 g filament. After surgery, start the tests with the 0.4 g filament. If a positive response is observed with the first tested filament, test a filament of lower force (instead of greater) at step 4.3.2.
    2. Apply the same filament to the next animals according to the 4.3.1 procedure. Once all animals are tested, start again on the first animal with the next filament of greater force. Repeat the procedure until all mice give a positive response. Notes: Test each animal

until two consecutive filaments give a positive response. Consider the gram value of the lower filament that gave a positive response as the paw withdrawal threshold for this animal.

Representative Results

The data are expressed as mean ± SEM. Statistical analyses were performed using multi-factor analysis of variance (ANOVA) or unpaired *t*-tests in accordance with the experimental design. For these analyses, the Sham and Cuff surgery groups as well as the saline vs. drug treatments were considered as between-group factors. When appropriate, repeated measure analyses were used for the time course data. The post-hoc comparisons were performed using the Duncan test. Statistical significance was considered at $p<0.05$.

When using the procedures that are described above, the cuff implantation results in an ipsilateral allodynia as illustrated in **Figure 1**. Once the mouse is habituated to the testing procedure, the values for paw withdrawal thresholds in the von Frey test remain stable over time and are not affected by the surgical procedure *per se*, as illustrated in Sham animals. It should however be noted that a transitory post-surgical allodynia can usually be observed in Sham mice. When such allodynia is present, the paw withdrawal response returns to baseline after a few days post-surgery. In Cuff mice, the ipsilateral allodynia is already present on the first days post-surgery and is maintained for more than 2 months (see **9**, and **Figure 1**; $F_{8,344}=29.5$, $p<0.001$). The cuff-induced allodynia remains ipsilateral in C57BL/6J mice when it is measured by the von Frey test as described above, but in other conditions a presence of allodynia on the contralateral paw can also be observed[8]. The absolute values for baseline are usually between 4 and 6 g in C57BL/6J mice, but the testing protocol may affect these values.

Tricyclic antidepressants are among clinical first-line treatments for neuropathic pain. In this model, the tricyclic antidepressant drug nortriptyline (5 mg/kg, intraperitoneal, twice a day) relieves the neuropathic allodynia after around 2 weeks of treatment, as illustrated in **Figure 2** ($F_{7,91}=15.3$, $p<0.001$; *post-hoc:* (CuffNor=Sham)>CuffSal at $p<0.001$ on days 29 - 34). At this dose, no acute analgesic action of the antidepressant is observed[16,17]. To mimic the lasting pain relief that is present in patients taking such drugs, the mice can be tested before the morning drug administration rather than after. Such procedure allows assessment of a long-lasting effect primed by previous days of treatment. In this case, it requires 1 to 2 weeks of treatment to observe a lasting relief of the neuropathic allodynia. When the treatment is interrupted, a relapse is usually observed within 3 to 4 days[18]. Beside some antidepressants, gabapentinoids are the other first-choice treatments for neuropathic pain. Gabapentin has an acute and transitory analgesic action in this model[16], but it also displays a delayed and long-lasting antiallodynic action when testing the animal each day before the drug administration (**Figure 3**; $p<0.001$). This action is faster than with antidepressant drugs.

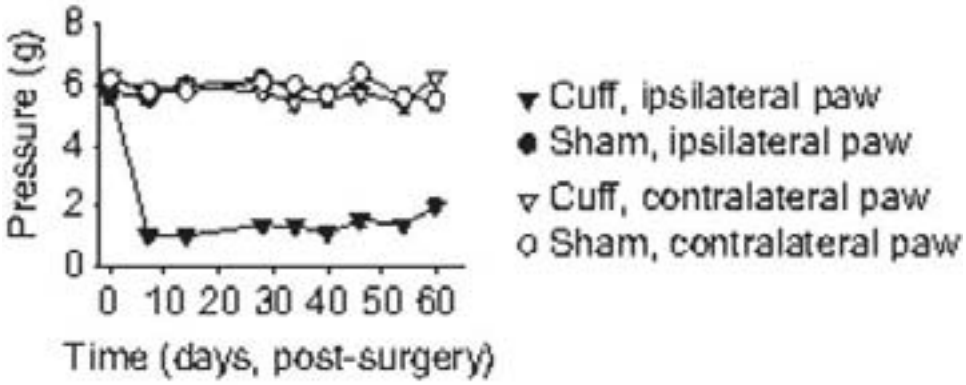

Fig. 1

Figure 1. Mechanical paw withdrawal thresholds in the cuff model of neuropathic pain in mice. Adult male C57BL/6J mice were habituated to the von Frey procedure until a stable baseline was obtained (the baseline is represented at point 0 on the graph). Both paws were tested. The Cuff mice display ipsilateral mechanical allodynia as showed by the lowered paw withdrawal thresholds (n=10 per group).

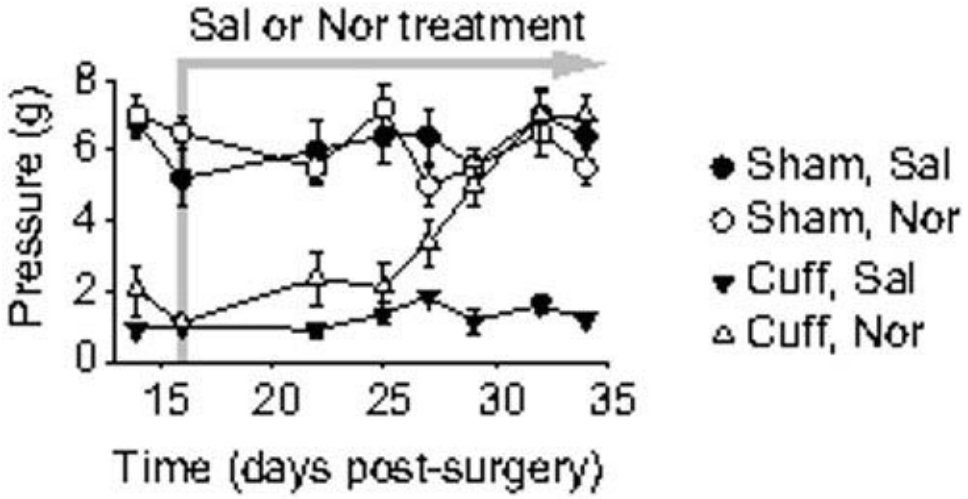

Fig.2

Figure 2. Delayed antiallodynic action of a tricyclic antidepressant. After two weeks post-surgery, mice received intraperitoneal treatment twice a day (morning and evening) with either 0.9% NaCl or 5 mg/kg nortriptyline hydrochloride (n=5 or 6 per group). The von Frey test was done before the morning treatment. With this procedure, a delayed antiallodynic action of nortriptyline is observed, which requires around 12 days of treatment.

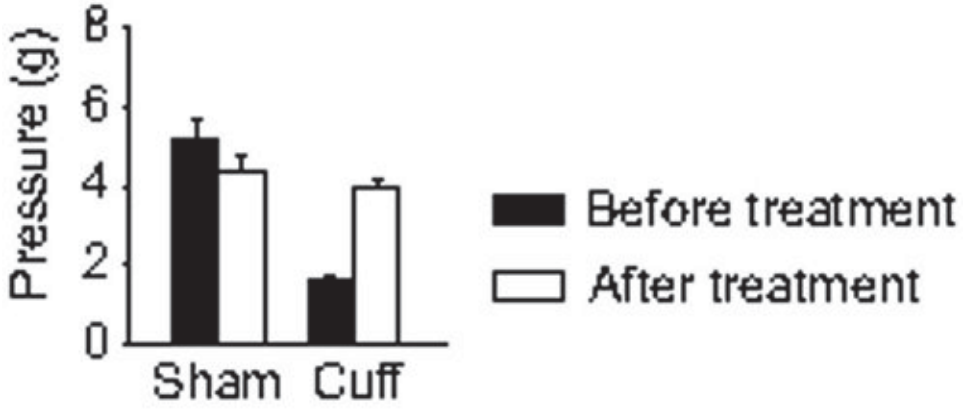

Fig.3

Figure 3. Antiallodynic action of a gabapentinoid. After three weeks post-surgery, mice received intraperitoneal treatment twice a day (morning and evening) with either 0.9% NaCl or 10 mg/kg gabapentin (n=5 per group). The von Frey test was done before the morning treatment. With this procedure, a delayed and lasting antiallodynic action of gabapentin is observed. Data are presented before starting the treatments and at the 6th day of treatments.

Discussion

The "cuff" model was initially developed in rats to obtain a standardized and reproducible chronic constriction injury with the implantation of multiple cuffs around the sciatic nerve[6]. It was then modified to implant a single cuff[7,8], even though some research groups still use multiple cuff insertion[19-22]. It was then adapted to mice[9,23], which opened the possibility to use transgenic animals. The cuff is usually 2 mm long, but other lengths have also been used in rats[22]. The polyethylene tubing depends on the species: PE-20 in mice[9], and PE-60[24,25] or PE-90[7,8,26,27] in rats.

The mechanical allodynia is measured with von Frey hairs. In this test, the absolute values for paw withdrawal thresholds may depend upon the surface on which the animal stands[28] or upon the duration of filament bending[3], but these factors do not affect the detection of the neuropathic allodynia.

The "cuff" model is of interest for the study of neuropathic pain mechanisms. It was used to study morphological changes in myelinated and unmyelinated fibers[6,29], and functional changes in sensory neurons, primary afferents and spinal neurons[19,21,22,30-35]. It allowed demonstration that glial activation and a central shift in neuronal anion gradient participate in changes in the activity and in the responses of spinal nociceptive neurons and in neuropathic allodynia[24,36-38]. The influence of glutamate receptors[7,39-41], of opioid receptors[16,42-45] and of nicotinic receptors[46] was also studied in this model.

Another interest of the model is its response to current treatments of neuropathic pain, *i.e*., gabapentinoids and antidepressants. Similar to clinical observations: gabapentinoids display both an acute short-lasting analgesic action at high dose and a delayed sustained relieving action that is observed after a few days of treatment, tricyclic antidepressants and selective serotonin and noradrenaline reuptake inhibitors have no acute analgesic effect at relevant dose but they display a delayed sustained relieving action that requires 1 to 2 weeks of treatment, and the selective serotonin reuptake inhibitor fluoxetine is ineffective[16]. The model is thus appropriate to study the molecular mechanism underlying these treatments[16-18,44,45,47], which may reveal new therapeutic targets to test in patients[48-51].

Lastly, the model also allows the study of the anxiodepressive consequences of neuropathic pain. Clinically, these consequences affect around a third of neuropathic pain patients but are preclinically less studied than the sensory aspects of pain. In this model, a time-dependent development of anxiety-like and depressive-like phenotypes is present[52] and the related mechanism can thus be addressed.

The standardized cuffs and procedures in this mouse model of neuropathic pain result in low interindividual variability for the mechanical allodynia. The possibility to use genetically modified animals[17,18,44-47,52], the long-lasting allodynia, the response to clinically used treatments and the time-dependent development of anxiodepressive symptoms make this model appropriate for the study of the various aspects and consequences of neuropathic pain and its treatments, which have already brought valuable information to this field of research.

Disclosures

The authors declare that they have no competing financial interests.

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Materials List for:

The Sciatic Nerve Cuffing Model of Neuropathic Pain in Mice

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Materials

Name	Company	Catalog Number	Comments
PE-20 polyethylene tubing	Harvard Apparatus	PY2-59-8323	Splitted before surgery
Ketamine	Centravet	IMA004	
Xylazine HCl	Sigma	X1251	Freshly prepared before surgery
Ocry-gel	Centravet		
Pliers	FST	11003-12	52.5 mm straight
Bulldog clamp	FST	p130 18038-45	
Perforated plate	CTTM		
von Frey filaments	Bioseb	NC-12775	

II. Antidepressants suppress neuropathic pain by a peripheral β_2-adrenoceptor mediated anti-TNFα mechanism.

Bohren Y, Tessier LH, Megat S, Petitjean H, Hugel S, Daniel D, **Kremer M**, Fournel S, Hein L, Schlichter R, Freund-Mercier MJ, Yalcin I, Barrot M. ***Neurobiol Dis***, 2013, 60:39-50.

Certains antidépresseurs sont actuellement des traitements cliniques de référence pour les douleurs neuropathiques. Les cibles primaires communes à ces antidépresseurs sont les sites de recapture de la sérotonine et de la noradrénaline, mais les molécules ciblant sélectivement la sérotonine sont peu efficaces face à la douleur neuropathique (Benbouzid et al., 2008a; Attal et al., 2010), suggérant un rôle préférentiel du système noradrénergique. Les travaux précédents de l'équipe suggéraient que la noradrénaline, indirectement recrutée par ces antidépresseurs, agit préférentiellement sur les β_2-AR pour soulager un des symptômes de la douleur neuropathique, l'allodynie mécanique (Yalcin et al., 2009a; Yalcin et al., 2009b). Il a aussi été montré que des agonistes sélectifs des β_2-AR avaient, chez l'animal, une action antiallodynique similaire à celle des antidépresseurs (Choucair-Jaafar et al., 2009; Yalcin et al., 2010). Ces résultats suggèrent un rôle bénéfique potentiel des β_2-mimétiques sur la douleur neuropathique, aspect exploré depuis par des études cliniques préliminaires (*cf. Avant-propos*).

Malgré ces avancées récentes et le fait que l'action thérapeutique des antidépresseurs soit bien documentée dans le domaine de la dépression, le ou les mécanisme(s) précis par le(s)quel(s) ces molécules atténuent la douleur neuropathique reste(nt) pour l'heure assez mal connu(s). Ce travail conduit par le Dr. Yohann Bohren, un doctorant de l'équipe, et publié dans *Neurobiology of Disease* apporte des compléments d'information quant à la localisation neuroanatomique du site d'action de la nortriptyline et quant au rôle d'éléments neuroinflammatoires dans son action sur la douleur neuropathique.

L'importance de la noradrénaline dans l'action thérapeutique des antidépresseurs est supportée par diverses études cliniques et précliniques (Krell et al., 2005; Attal et al., 2006; Yalcin et al., 2009a; Attal et al., 2010; Dharmshaktu et al., 2012). La noradrénaline peut être libérée au sein de structures supraspinales (Pertovaara, 2006; El Mansari et al., 2010; Pertovaara, 2013; Llorca-Torralba et al., 2016) et spinales, par les projections issues

principalement du locus cœruleus (Yoshimura and Furue, 2006; Llorca-Torralba et al., 2016), ou au niveau périphérique par les efférences sympathiques qui forment des bourgeonnements dans les ganglions rachidiens suite à une lésion des nerfs périphériques (McLachlan et al., 1993; Ramer and Bisby, 1998). Identifier la source de noradrénaline requise dans l'action thérapeutique des antidépresseurs est une étape clé dans l'étude de leur mécanisme d'action sur la douleur neuropathique. Grâce à des lésions noradrénergiques ciblées, centrales et / ou périphériques, nous avons montré que le site d'action primaire de l'effet de la nortriptyline dans le soulagement de l'allodynie neuropathique périphérique est le ganglion rachidien. De plus, par une approche d'imagerie calcique, nous avons montré que l'action de la noradrénaline s'effectue par les β_2-AR majoritairement exprimés par les cellules non neuronales des ganglions rachidiens.

Enfin, ces dernières années, l'hypothèse d'une implication de cellules immunocompétentes et gliales dans la physiopathologie des douleurs neuropathiques est de plus en plus envisagée dans la littérature (Marchand et al., 2005; Leung and Cahill, 2010). Le deuxième objectif de cet article a donc été d'étudier les changements d'expression de médiateurs neuroimmunitaires, en particulier des cytokines, dans notre modèle murin de douleur neuropathique périphérique. Ainsi, nous avons mis en évidence que l'expression du TNFα, en particulier sa forme membranaire (mTNFα), est augmentée dans les ganglions rachidiens en condition neuropathique et qu'un traitement prolongé par des antidépresseurs, la nortriptyline ou la venlafaxine, diminue cette surexpression, suggérant ainsi que l'effet antiallodynique de ces antidépresseurs impliquerait une diminution d'expression du TNFα majoritairement exprimé par les cellules non neuronales du ganglion rachidien. Suite à ces résultats, j'ai évalué l'action de médicaments anti-TNFα utilisés en clinique, principalement dans les pathologies inflammatoires telles que la polyarthrite rhumatoïde ou la spondylarthrite ankylosante, l'etanercept (Enbrel®) et l'infliximab (Remicade®), sur l'allodynie mécanique. Les résultats ont montré une action antiallodynique de ces deux médicaments soulignant, là encore, l'importance de cette cytokine pro-inflammatoire dans la physiopathologie de la douleur neuropathique.

Ces résultats suggèrent que l'effet thérapeutique d'un traitement prolongé par les antidépresseurs impliquerait la noradrénaline en provenance du système sympathique périphérique, les β_2-AR des cellules non neuronales des ganglions rachidiens et conduirait à une régulation d'expression d'une cytokine pro-inflammatoire, le TNFα. Dans cette étude,

ma contribution a été d'évaluer l'efficacité des deux traitements anti-TNFα, l'etanercept et l'infliximab, sur l'allodynie mécanique neuropathique (**Figure 5 de l'article**). J'ai également participé à l'obtention des données histologiques montrant que le TNFα est majoritairement exprimé par les cellules non neuronales des ganglions rachidiens (**Figures 6 et 7 de l'article**).

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Antidepressants suppress neuropathic pain by a peripheral β2-adrenoceptor mediated anti-TNFα mechanism☆

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ABSTRACT

Neuropathic pain is pain arising as a direct consequence of a lesion or disease affecting the somatosensory system. It is usually chronic and challenging to treat. Some antidepressants are first-line pharmacological treatments for neuropathic pain. The noradrenaline that is recruited by the action of the antidepressants on reuptake transporters has been proposed to act through β2-adrenoceptors (β2-ARs) to lead to the observed therapeutic effect. However, the complex downstream mechanism mediating this action remained to be identified. In this study, we demonstrate in a mouse model of neuropathic pain that an antidepressant's effect on neuropathic allodynia involves the peripheral nervous system and the inhibition of cytokine tumor necrosis factor α (TNFα) production. The antiallodynic action of nortriptyline is indeed lost after peripheral sympathectomy, but not after lesion of central descending noradrenergic pathways. More particularly, we report that antidepressant-recruited noradrenaline acts, within dorsal root ganglia, on β2-ARs expressed by non-neuronal satellite cells. This stimulation of β2-ARs decreases the neuropathy-induced production of membrane-bound TNFα, resulting in relief of neuropathic allodynia. This indirect anti-TNFα action was observed with the tricyclic antidepressant nortriptyline, the selective serotonin and noradrenaline reuptake inhibitor venlafaxine and the β2-AR agonist terbutaline. Our data revealed an original therapeutic mechanism that may open novel research avenues for the management of painful peripheral neuropathies.

Introduction

Neuropathic pain is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system (Jensen et al., 2011). This complex syndrome implicates maladaptive changes in injured sensory neurons and along the entire nociceptive pathway within the central nervous system (von Hehn et al., 2012). In the clinic, tricyclic antidepressants (TCAs) as well as selective serotonin and noradrenaline reuptake inhibitors are recommended among first-line treatments (Attal et al., 2006, 2010; Dworkin et al., 2007; Saarto and Wiffen, 2007). In contrast, selective serotonin reuptake inhibitors are poorly effective (Attal et al., 2006; Benbouzid et al., 2008a; Dworkin et al., 2007), suggesting that the noradrenergic component of antidepressants has a key role in their action on neuropathic pain. Despite the fact that the therapeutic effect of antidepressants is well documented, the precise mechanism by which neuropathic pain is alleviated remains poorly understood (Mico et al., 2006).

Sustained antidepressant treatment is necessary to be effective against neuropathic pain, suggesting the recruitment of an indirect mechanism. Pharmacological and genetic approaches showed that antidepressant-recruited noradrenaline selectively acts through β2-adrenoceptors (β2-ARs) to relieve neuropathic allodynia (Yalcin et al., 2009a, 2009b), and that repeated stimulation of these receptors by direct agonists is sufficient to reach a therapeutic effect (Choucair-Jaafar et al., 2009, 2011; Yalcin et al., 2010). A clinical case report appears to support the action of β2-mimetics against neuropathic pain (Cok et al., 2010).

To progress into the pain relief mechanism, it is important to identify its neuroanatomical substrate. A critical step is to determine the source of noradrenaline recruited by antidepressants. Indeed, noradrenaline

Abbreviations: *Arbp*, acidic ribosomal phosphoprotein P0; β2-AR, β2-adrenoceptor; CGRP, calcitonin gene related peptide; GS, glutamine synthetase; IL, interleukin; NF200, neurofilament 200; NP, neuropathic pain group; TCA, tricyclic antidepressant; TNFα, tumor necrosis factor α; TH, tyrosine hydroxylase.

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can be released within supraspinal structures (El Mansari et al., 2010), at the spinal level by descending noradrenergic pathways (Millan, 2002; Yoshimura and Furue, 2006), and at the peripheral level in dorsal root ganglia following neuropathy-induced noradrenergic sprouting of sympathetic nerve fibers (McLachlan et al., 1993; Ramer and Bisby, 1998). Previous data with local administration of a β2-AR antagonist suggested that the initial substrate for antidepressants' antiallodynic action might be localized at the spinal cord and/or at the dorsal root ganglia level rather than at the supraspinal level (Yalcin et al., 2009b).

Although β2-ARs are critical for the antiallodynic action of antidepressants, the downstream mechanism has not yet been identified. Experimental evidence supports a role of glial and/or immune cells in the pathophysiology of neuropathic pain, particularly through the recruitment of cytokines (Austin and Moalem-Taylor, 2011; Thacker et al., 2007; Vallejo et al., 2010). Clinical studies also support cytokine implication in neuropathic pain (Empl et al., 2001; Lindenlaub and Sommer, 2003). It is however not known whether antidepressant treatment targets these neuroimmune actors.

In this study, we demonstrate that the peripheral nervous system is essential for the antiallodynic effect of nortriptyline. We show that the noradrenaline recruited by this antidepressant acts within dorsal root ganglia, on β2-ARs expressed by non-neuronal satellite cells. We also show that both nortriptyline and venlafaxine inhibit local TNFα production. These findings reveal a novel cellular and molecular substrate for the antiallodynic action of antidepressants.

Material and methods

Animals

Experiments were done using male C57BL/6J mice (Charles River, L'Arbresle, France), or with mice lacking β2-ARs and their littermate controls. In the latter case, the mice were created in the laboratory of Brian Kobilka (Stanford University, CA) and have been described previously (Chruscinski et al., 1999). Heterozygotes (*Adrb2*$^{+/-}$) were bred in our animal facilities, mice were genotyped upon weaning, and the experiments were conducted on male *Adrb2*$^{+/+}$ and *Adrb2*$^{-/-}$ littermate mice. All experiments started with 8- to 9-week-old mice. They were group-housed four to five per cage and kept under a 12-hour light/dark cycle (lights on at 6:00 AM) with food and water *ad libitum*. All animals received proper care in agreement with the guidelines for animal experimentation of the International Association for the Study of Pain and the European Communities Council Directive 86/6609/EEC. The animal facilities are registered for animal experimentation with approved procedures by Alsace regional veterinary office (agreement C67-482-1) and the scientists in charge of the experiments possess the French legal certificate authorizing experimentation on living animals.

Neuropathy and nociceptive testing

The neuropathy was induced by cuffing the main branch of the right sciatic nerve (Benbouzid et al., 2008b; Mosconi and Kruger, 1996). Surgeries were done under ketamine (17 mg/mL)/xylazine (2.5 mg/mL) anesthesia (i.p., 4 mL/kg) (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 (neuropathic pain group: NP). The shaved skin was closed using suture. Sham-operated mice underwent the same surgical procedure without implantation of the cuff (Sham group). The mechanical allodynia was tested using von Frey hairs and results were expressed in grams. Mice were placed in clear Plexiglas boxes (7 cm × 9 cm × 7 cm) on an elevated mesh screen. Calibrated von Frey filaments (Bioseb, Vitrolles, France) were applied to the plantar surface of each hindpaw until they just bowed, in a series of ascending forces up to the mechanical threshold. Each filament was tested five times per paw and the threshold was defined as the lower of two consecutive filaments for which three or more withdrawals out of the five trials were observed (Barrot, 2012; Bohren et al., 2010). To study the antiallodynic effect of chronic drug treatments, the mice were tested in the morning, before drug injections when applicable, thus reflecting the therapeutic action of previous treatment days, as previously described (Bohren et al., 2010; Yalcin et al., 2009a, 2009b).

Treatment procedures

Treatments with the TCA nortriptyline, the selective serotonin and noradrenaline reuptake inhibitor venlafaxine, the β2-mimetic terbutaline, or the anti-TNFα etanercept or infliximab, began fifteen or sixteen days after the neuropathy was induced, and they were maintained for either 2 or 3 weeks. During the treatments, the mice received two injections per day (morning and evening) of nortriptyline (5 mg/kg), venlafaxine (5 mg/kg) or terbutaline (0.5 mg/kg) (Sigma-Aldrich, Saint-Quentin Fallavier, France), one injection per day (morning, 5 mg/kg) of etanercept (ENBREL®, Wyeth Pharmaceuticals, Blois, France), one injection every other day (2 mg/kg) of etanercept, or one unique injection of infliximab (10 mg/kg, REMICADE®, Schering-Plough, Levallois-Perret, France). Drugs were dissolved in 0.9% NaCl solution that was also used for control injections, and they were administered intraperitoneally in a volume of 5 mL/kg. For cellular and molecular experiments, the nervous tissues were collected five weeks post-surgery, *i.e.* after three weeks of treatment.

Noradrenergic lesions

Chemical noradrenergic lesion was done using guanethidine monosulfate (ISMELIN®, Laboratoires Genopharm, Saint-Thibault-des-Vignes, France) (Neil et al., 1991). Three different paradigms were used, each targeting different anatomical sites. (1) A group of mice underwent five daily intraperitoneal injections of guanethidine (30 mg/kg) in a volume of 5 mL/kg; (2) another received three daily intrathecal injections at the lumbar level (30 μg in 10 μL); and (3) a last group received three daily intrathecal injections at the thoracic level (30 μg in 10 μL). Intrathecal injections were performed under gaseous anesthesia (halothane 3%). Briefly, for lumbar intrathecal administration, a 27-gauge needle connected to a 50 μL Hamilton syringe was inserted between the L5 and L6 vertebrae, into the sub-arachnoidal space. Placement of the needle was verified by the elicitation of a tail flick movement. For thoracic administration, the needle was inserted at the level of the last thoracic vertebrae, just above the lower rib used as mark. All guanethidine administrations were done two weeks before nerve injury. A control group of non-lesioned mice underwent the same nerve cuffing procedure as the lesioned groups.

Immunostaining

Five weeks post-surgery, neuropathic and sham mice were deeply anesthetized with pentobarbital and perfused intracardially. Lumbar dorsal root ganglia (L4, L5 and L6) and spinal cord were dissected, postfixed, cryoprotected, embedded in OCT compound (Sakura Finetek, Villeneuve d'Ascq, France), frozen and cut into 14 μm thick sections that were mounted on Superfrost®Plus slides (O. Kindler GmbH, Freiburg, Germany). To evaluate tyrosine hydroxylase (TH) expression, we used standardized procedures (Kaufling et al., 2010), incubating the sections with Sheep anti-mouse TH antibody (1:500, Millipore, AB1542, France Millipore, Molsheim, France). The sections were then incubated with secondary Cy3-conjugated antibodies done in donkey (1:400, Jackson ImmunoResearch, Newmarket, UK). Thereafter, the sections were washed, mounted with VECTASHIELD, and viewed under a Nikon E80i microscope with ×10 and ×20 objectives, and images acquired with MBF Bioscience camera CX9000 using Picture Frame acquisition software (MBF Bioscience, Magdeburg, Germany). For TNFα detection, mice

received an acute infliximab injection (10 mg/kg, i.p.) at least two weeks after sciatic nerve surgery, and were perfused 6 h post-injection. Cy3-conjugated anti-human antibodies (Jackson ImmunoResearch, 709-165-149) were used to detect infliximab. Costaining was done either by using Rabbit anti-glutamine synthetase (GS) (1:500, Sigma Aldrich, G2781) for satellite cells, NeuroTrace™ fluorescent Nissl for neurons (1:200, Molecular Probes, N-21479), or by using Rabbit anti-calcitonin gene related peptide (CGRP) (1:20,000, Amersham, RPN1842), Rabbit anti-neurofilament 200 (NF200) (1:2000, Sigma Aldrich, N4142) or Rabbit anti-substance P (1:15,000, gift from Dr. Tramu, Bordeaux) antibodies, or isolectin IB_4 Alexa Fluor® 488 conjugate (2 μg/mL, Molecular Probes, I21411) for staining of different neuronal cell populations. When an additional fluorescent secondary antibody was required, we used Alexa Fluor® 488-conjugated antibodies (Molecular Probes). Microphotographs for detection of infliximab only were done with a Nikon E80i microscope with ×4, ×10 and ×40 objectives, and images acquired with MBF Bioscience camera CX9000 using Picture Frame acquisition software. Microphotographs for fluorescence double labeling were taken using a Leica confocal microscope TCS SP5 II, under ×20 objective, and analyzed using the Leica application suite advanced fluorescence software. For all pictures, Adobe Photoshop CS3 (Adobe, San José, CA, USA) was used to adjust contrast and brightness.

Dorsal root ganglia cell culture

Dorsal root ganglia cells were prepared from adult mice. Dorsal root ganglia from lumbar levels were dissected out, collected in phosphate-buffered solution, and enzymatically dissociated for 25 min at 37 °C with trypsin–EDTA (0.5 g/L, Seromed) and 2 mg/mL of collagenase IA/dispase (Invitrogen). Enzymatic dissociation was stopped by the addition of the culture medium which consisted of MEMα (minimum essential medium alpha, Gibco, France) supplemented with 10% v/v heat-inactivated horse serum (Gibco) and 50 IU/mL penicillin–streptomycin (Gibco). The cells were then mechanically dissociated by trituration with fire-polished Pasteur pipettes of decreasing tip diameter. Dorsal root ganglia cells were plated on 15 mm glass coverslips previously coated with poly-D-lysine (0.02 mg/mL). Cultures were maintained at 37 °C in a water-saturated atmosphere (95% air and 5% CO_2). Cell cultures were suitable for single cell PCR or calcium imaging experiments and were respectively used between 2 and 5 h or between 12 and 15 h after the seeding.

Single cell PCR

Single-cell PCR experiments were conducted on dissociated dorsal root ganglia cell culture of naïve mice. Cells were collected individually from the coverslip using a patch clamp pipette filled with 8 μL of buffer solution (mM): 140 KCl, 1 $MgCl_2$, 0.5 EGTA, pH 7.4. The glass pipette tip was then broken off into a thin-walled PCR reaction tube containing 40 μL of the reaction mix SuperScriptIII One-Step RT-PCR system (Invitrogen) and the first set of primers. This first set included couples of primers for NF200 (accession number: NM_010904), β2-AR (accession number: NM_007420) and GS (accession number: AY044241). 2 μL of SuperScriptIII RT/Platinum Taq mix was added to each sample and cDNAs were synthesized by incubation at 50 °C for 30 min and the first PCR consisted of an initial denaturation at 94 °C for 2 min followed by 37 cycles (94 °C for 30 s, 52 °C for 45 s, 68 °C for 45 s) and a final extension at 68 °C for 5 min. The resulting product was diluted 1:100 and re-amplified by Taq PCR (Invitrogen) using a single couple of nested primers for each specific gene studied. The second amplification consisted of an initial denaturation step at 94 °C for 2 min followed by 35 cycles (94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s) and a final extension at 72 °C for 5 min. One-fifth of the PCR product was run on an agarose gel and stained with ethidium bromide.

Calcium imaging

Calcium imaging experiments were conducted on dissociated dorsal root ganglia cell culture of sham and neuropathic mice. During calcium measurements, the cells were continuously superfused with a saline solution containing (mM): 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 5 HEPES, pH 7.4. High KCl solution had the same composition, except: KCl, 50 mM and NaCl, 90 mM. The whole dish was perfused with saline solution, and the imaged field was perfused by a single-tip multichannel gravity-fed system, allowing a switch between various perfused solutions. Cells were loaded with Fura-2 by 60-minute incubation at room temperature in the recording solution with 2 μM Fura-2 acetoxymethyl ester (Fura-2/AM; Molecular Probes) and 0.001% (w/v) pluronic acid (Molecular Probes). Cells were washed three times with saline solution before and after loading. Fluorescence measurements on individual cells were performed on an inverted microscope (Axiovert 35; Zeiss) with an oil-immersion ×40 objective (Fluor 40, NA 1.30; Nikon) using a quantitative real-time imaging system comprising a cooled CCD camera (CoolSnap HQ, Photometrics) and an image analysis software package (Imaging Workbench 4.0 Software, Axon Instruments). Fluorescence was alternately excited at wavelengths of 350 and 380 nm with a lambda-10 filter wheel (Sutter Instruments), and emitted light was collected above 520 nm. Pairs of images were acquired every 2 s. Intracellular calcium is expressed throughout as the fluorescence ratio F_{350}/F_{380}, calculated after background subtraction. Experiments were performed at room temperature. All drugs were prepared as 1000× concentrated stock solutions. ATP (50 μM), ADP (50 μM), UTP (50 μM) and terbutaline (100 μM) were diluted into saline solution immediately before use.

Real-time quantitative PCR

Quantification of gene expression in dorsal root ganglia in each group was assessed with a real-time quantitative PCR method. Total RNA was extracted using RNeasy (QIAGEN), and cDNA was generated with *High capacity RNA-to-cDNA master mix* from Applied Biosystems. Quantitative real-time PCR was performed on a StepOne Real-Time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix assay (Applied Biosystems). The amplification reaction was performed for 40 cycles with denaturation at 95 °C for 10 min, followed by annealing at 95 °C for 15 s, and extension and detection at 60 °C for 1 min. All experiments were done with triplicate sample deposits on the amplification plates. The relative RNA abundance of each target gene transcript was normalized against endogenous gene control acidic ribosomal phosphoprotein P0 (*Arbp*). Data were analyzed according to relative gene expression by $2^{-\Delta\Delta Ct}$ method, where Ct represents the threshold cycle for each transcript (Schmittgen and Zakrajsek, 2000). Primers (designed from *Mus musculus* gene data bank using Applied Biosystems Primer Express Software version 3.0) are as follows: *Arbp* (or *Rp1p0*, acidic ribosomal phosphoprotein): 5′-GCCAGCTCAGAACAC TGGTCTA-3′ and 5′-ATGCCCAAAGCCTGGAAGA-3′; *Tnf* (TNFα, tumor necrosis factor): 5′-CAGCCGATGGGTTGTACCTT-3′ and 5′-GGCAGCCTT GTCCCTTGA-3′; *Il10* (IL-10, interleukin10): 5′-GATGCCCCAGGCAGAG AA-3′ and 5′CACCCAGGGAATTCAAATGC-3′; *Tnfrsf1a* (TNF-R1, tumor necrosis factor receptor 1): 5′-TCCGCTTGCAAATGTCACA-3′ and 5′-GGCAACAGCACCGCAGTAC-3′; *Adbr2* (β2-AR): 5′-TGGGAACGGCTACTC TAGCAA-3′ and 5′-TGTTTGGCTCCCCTGTGTAGT-3′; *Ptgs2* (Cox-2, prostaglandin G/H synthase 2): 5′-TTCGGGAGCACAACAGAGTGT-3′ and 5′-GCTCATCACCCCACTCAGGAT-3′; *Il1a* (IL-1α, interleukin 1 alpha): 5′-GGAGAGCCGGGTGACAGTATC-3′ and 5′-TCAGCCGTCTCTTCTTCAGAAT C-3′; Il1B (IL-1β, interleukin 1 beta): 5′-AGTTGACGGACCCCAAAAGA-3′ and 5′-GGACAGCCCAGGTCAAAGG-3′; and *Il6* (IL-6, interleukin 6): 5′-CCACGGCCTTCCCTACTTC-3′ and 5′-TTGGGAGTGGTATCCTCTGTGA-3′.

Two procedures were used depending on the set of experiments. One was done with a total of n = 9 mice per group (Fig. 4B). To limit

potential interindividual differences due to either mice or the dissection procedure, we pooled samples from the 3 lumbar dorsal root ganglia of 3 mice from the same experimental group (*i.e.* 9 dorsal root ganglia). These pooled samples were processed for real-time PCR, each sample being present as triplicate on the plate. We repeated the experiment two other times, with pooled sets of 3 other animals each time. The other sets of experiments were conducted on samples from individual mice. All individual samples were compared to a reference composed of a mix of the controls' cDNAs and results were standardized by the $2^{-\Delta\Delta Ct}$ method as described above.

Immunoblotting analysis

Due to differences in optimized extraction procedures, real-time quantitative PCR and Western blot were always done on independent sets of mice. For Western blot, total protein was extracted in 150 μL lysis buffer (20 mM Tris pH 7.5; 150 mM NaCl; 10% glycerol; 1% NP40; Protease Inhibitors Cocktail, Roche). The proteins were quantitated with DC protein assay kit (Bio-Rad) and 15 μg of total protein from individual animals was resolved by 12% SDS-polyacrylamide gel electrophoresis under reducing conditions and then transferred to PVDF membrane. The blots were incubated for 1 h in blocking agent (ECL kit, Amersham Biosciences) and subsequently, overnight with the specific antibodies for TNFα (1:1000, R&D Systems, AF-410-NA) and β-tubulin (1:5000, Santa Cruz Biotechnology Inc., sc-9935) followed by anti-goat HRP-conjugated secondary antibodies (1:5000, Chemicon, AP307P and AP106P). Blots were evaluated by chemiluminescence (ECL *Advance Western Blotting Detection System Kit*, Amersham Biosciences, RPN 2135) using Hyperfilm substrates (Amersham Biosciences, RPN 1674K). Relative protein expression was determined using the densitometry tool of Adobe Photoshop CS3 software.

ELISA assays

Commercially available ELISA reagents were used to evaluate concentrations of TNFα (BD OptEIA™, BD Biosciences, San Diego, CA). All procedures were performed following the manufacturer's instructions. Results were expressed as cytokine concentration in pg/mL. The detection limit was 15 pg/mL.

Statistics

Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed with STATISTICA 8 software (StatSoft, Tulsa, OK, USA), using multifactor 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 time of measurement (time course data) was taken as a within-subject factor. The Duncan test was used for post hoc comparisons. In calcium imaging experiments, the fraction of cells answering or not to terbutaline application in sham and neuropathic conditions were compared by a contingency table with an Exact Fischer's test. Finally, for quantitative PCR and immunoblotting experiments, statistical analyses were performed with a non-parametric test of Kruskal–Wallis and comparisons between groups were done by U Mann–Whitney test. The significance level was set at $p < 0.05$.

Results

Antidepressant action on neuropathic pain requires peripheral noradrenaline

To mimic human neuropathy resulting from a trauma of peripheral nerves, we used chronic sciatic nerve cuffing in mice (Benbouzid et al., 2008b; Mosconi and Kruger, 1996). Mechanical allodynia is one of the symptoms distressing the patients. In the neuropathic mice, the ipsilateral allodynia appears on the first day post-surgery and persists over three months (Benbouzid et al., 2008b). This allodynia is relieved by chronic treatment with the TCA nortriptyline or the β2-AR agonist terbutaline (Benbouzid et al., 2008a; Choucair-Jaafar et al., 2009) (Fig. 1) (Nortriptyline, right paw: $F_{7,140} = 3.77$, $p < 0.001$; post-hoc: NP-Nortriptyline > NP-Saline at $p < 0.0001$ on post-surgery days 28 to 35. Terbutaline, right paw: $F_{7,140} = 3.09$, $p < 0.005$; post-hoc: NP-Terbutaline > NP-Saline at $p < 0.0001$ on post-surgery days 28 to 35). As observed in other neuropathic pain models (McLachlan et al., 1993; Ramer and Bisby, 1998), the cuff insertion also induced a sprouting of noradrenergic fibers in the lumbar dorsal root ganglia (Fig. 2A).

Since preclinical (Yalcin et al., 2009a) and clinical (Krell et al., 2005) data showed that the noradrenergic system is essential for the therapeutic action of antidepressant drugs, we investigated the possible source of endogenous noradrenaline which is targeted by antidepressant drugs to alleviate neuropathic allodynia. For this purpose, noradrenergic lesions were done two weeks before induction of the neuropathy, with guanethidine which does not cross the blood–brain barrier (Neil et al., 1991). This drug enters noradrenergic terminals through the noradrenaline transporter. While guanethidine at low dose is clinically used for peripheral depletion of noradrenaline storage sites, larger doses of guanethidine lead to the selective destruction of sympathetic fibers. This depletion of noradrenergic fibers did not affect *per se* the basal mechanical sensitivity or the neuropathic allodynia (Fig. 2B). However, both the intraperitoneal and the lumbar intrathecal injections of guanethidine prevented the antiallodynic action of nortriptyline (Fig. 2C) (Intraperitoneal: $F_{7,175} = 2.72$, $p < 0.015$; post-hoc: (NP-Nortriptyline = NP-Saline) < Sham-Saline at $p < 0.0001$ on post-surgery days 3 to 35. Lumbar: $F_{7,175} = 2.47$, $p < 0.02$; post-hoc: (NP-Nortriptyline = NP-Saline) < Sham-

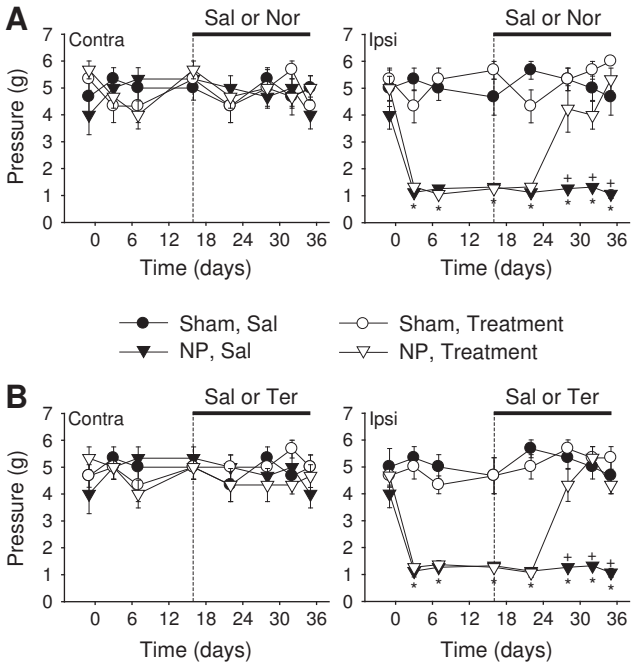

Fig. 1. Chronic nortriptyline or terbutaline treatments relieve neuropathic allodynia. Two weeks after nerve injury (NP, neuropathic pain group), treatment with the TCA nortriptyline (Nor, 5 mg/kg, twice a day), the β2-AR agonist terbutaline (Ter, 0.5 mg/kg, twice a day) or their saline control (0.9% NaCl) started and was maintained for 3 weeks. The hindpaw mechanical threshold was tested using von Frey filaments. (A) Long-term nortriptyline treatment did not affect the threshold of the contralateral paw, but it suppressed the ipsilateral neuropathy-induced allodynia. (B) Similar results were observed with terbutaline. (Data from one experiment with $n = 6$ mice per group, $^{*}p < 0.001$ *vs.* Sham, $^{+}p < 0.05$ NP-treated *vs.* NP-Saline.) Data are expressed as mean ± SEM.

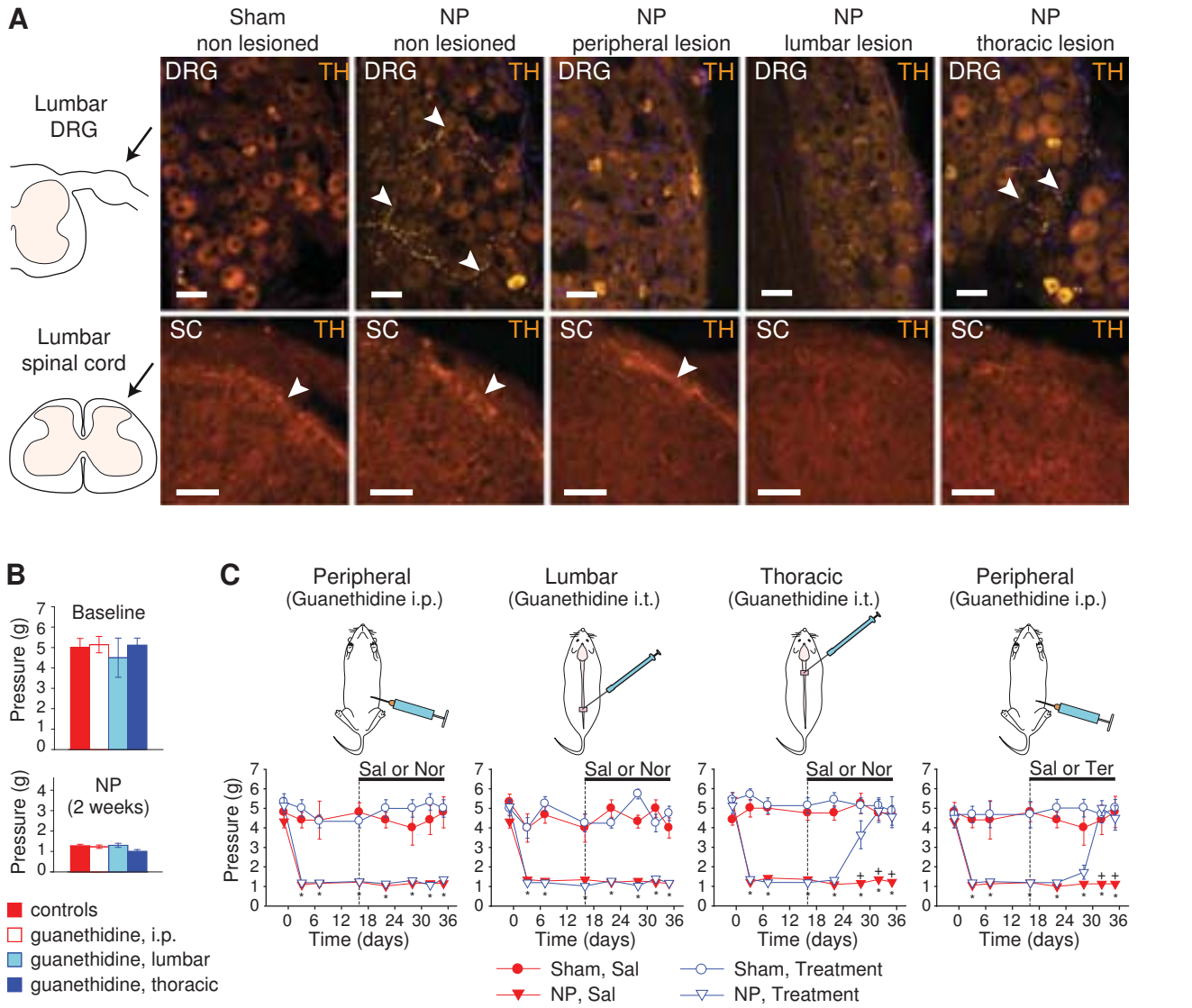

Fig. 2. Peripheral noradrenergic fibers mediate allodynia relief by antidepressant drug. Guanethidine injections allowed lesioning the noradrenergic fibers. (A) Tyrosine hydroxylase (TH) immunostaining in the lumbar dorsal root ganglia (DRG; scale bars: 100 μm) and the spinal cord (SC; scale bars: 50 μm). The peripheral neuropathy (NP) induced a sympathetic sprouting in the DRG (white arrows). Intraperitoneal guanethidine (peripheral lesion) suppressed these fibers without affecting the spinal cord ones. Lumbar intrathecal guanethidine suppressed both DRG and dorsal horn noradrenergic fibers. Thoracic intrathecal guanethidine suppressed descending noradrenergic fibers, sparing DRG sprouting. (B) Two weeks after guanethidine injections, no difference was observed between control mice and mice that received intraperitoneal, lumbar intrathecal or thoracic intrathecal guanethidine (baseline, top graph). Similarly, these injections of guanethidine did not affect the neuropathic allodynia (NP at 2 weeks, bottom graph). (C) The peripheral and the lumbar intrathecal injections of guanethidine, but not the thoracic intrathecal one, prevented the antiallodynic action of the TCA nortriptyline (Nor). The antiallodynic action of the β2-AR agonist terbutaline (Ter) was still present in mice which received peripheral guanethidine. (Data pooled from 2 independent experiments, $n = 5$–9 per group, $^{*}p < 0.001$ *vs.* Sham, $^{+}p < 0.05$ NP-treated *vs.* NP-Saline).

Saline at $p < 0.0001$ on post-surgery days 3 to 35). While intraperitoneal guanethidine induced a sympathectomy, suppressing peripheral noradrenergic fibers only, the lumbar intrathecal administration affected both the spinal cord and noradrenergic fibers in the lumbar dorsal root ganglia nearby the guanethidine injection site (Fig. 2A). This suggests a critical role of dorsal root ganglia noradrenergic sprouting in the TCA action, but it does not exclude a contribution of spinal descending pathways. To address this question, spinal noradrenergic fibers were suppressed without affecting the lumbar dorsal root ganglia sprouting, by doing thoracic intrathecal administration of guanethidine. This led to suppression of descending noradrenergic fibers, including at the lumbar level (Fig. 2A). In this case the antiallodynic action of nortriptyline was maintained (Fig. 2C) (Thoracic: $F_{7,168} = 6.55$, $p < 0.0001$; post-hoc: NP-Nortriptyline > NP-Saline at $p < 0.005$ on post-surgery days 28 to 35). Together, these results indicate that noradrenergic sprouting in the dorsal root ganglia is a critical substrate for the antiallodynic action of a TCA.

In order to test the location of β2-ARs in the therapeutic mechanism, we tested terbutaline in sympathectomized mice. This β-mimetic remained effective in the absence of sympathetic fibers in the dorsal root ganglia (Fig. 2C) ($F_{7,147} = 4.14$, $p < 0.0005$; post-hoc: NP-Terbutaline > NP-Saline at $p < 0.0001$ on post-surgery days 32 and 35). It showed that the implicated β2-ARs were neither on noradrenergic terminals nor upstream of the noradrenergic system; thus confirming a downstream, postsynaptic role of β2-ARs.

β2-ARs are located on non-neuronal dorsal root ganglia cells

Antidepressant-recruited noradrenaline acts on β2-ARs to relieve neuropathic allodynia (Yalcin et al., 2009a, 2009b). To identify the type of cells expressing mRNA encoding β2-ARs in dorsal root ganglia, we performed single cell PCR on dissociated dorsal root ganglia cell culture (Fig. 3A). NF200 neurofilament was used as a neuronal marker (Ho and O'Leary, 2010) and GS as a marker of satellite glial cells in the

dorsal root ganglia (Hanani, 2005). About a third of the tested cells were positive for β2-AR mRNA, but they were also positive for both NF200 and GS (Fig. 3A), suggesting that individual dorsal root ganglia neurons could not be dissociated from the satellite cells tightly covering them (Hanani, 2005). We overcame this problem by using a functional approach with calcium imaging on dorsal root ganglia cell culture. High KCl was used as neuronal activator, a response to ADP and/or UDP in the absence of response to KCl allowed identifying non-neuronal cells, and terbutaline was used as selective β2-AR agonist. In sham mice, around 3% of non-neuronal cells expressed functional β2-ARs, whereas 25% of non-neuronal cells from neuropathic animals displayed a calcium response to terbutaline (Figs. 3B, C) (Fisher's exact test: $p < 0.002$). The pathological condition thus increased functional β2-ARs in dorsal root ganglia, without altering mRNA expression of these receptors (Fig. 3D). Contrasting with these non-neuronal cells, neurons from either sham or neuropathic mice never responded to terbutaline application (Fig. 3C), suggesting that functional β2-ARs increasing intracellular calcium concentrations are selectively located in the plasma membrane of non-neuronal cells.

Antidepressants and a β2-AR agonist display anti-TNFα action

Cytokines produced by non-neuronal cells, such as glial and immune cells, participate in the development and maintenance of neuropathic pain (Austin and Moalem-Taylor, 2011; Thacker et al., 2007; Vallejo et al., 2010).

By Western blot ($p < 0.001$) and mRNA analysis ($p < 0.05$), we observed increased levels of TNFα in the lumbar dorsal root ganglia of C57BL/6J neuropathic mice at 5–6 weeks post-injury (Fig. 4A). To further examine the role of neuroinflammatory mediators in the neuropathic pain treatment by antidepressants or by a β2-AR agonist, gene expression of TNFα, IL-10, Cox-2, IL-1 and IL-6 was analyzed in the lumbar dorsal root ganglia. The implication of some of these mediators is well established in the early phase following nerve injury (Lee et al., 2004; Uceyler et al., 2007). However, their role in the dorsal root ganglia is less clear at much later time-points, when chronic neuropathic pain is well installed. Five weeks post-injury, we observed a preferential role for local TNFα. Indeed, an increased mRNA expression of TNFα was observed in neuropathic mice (Fig. 4B) ($H_{3,12} = 9.72$, $p < 0.05$),

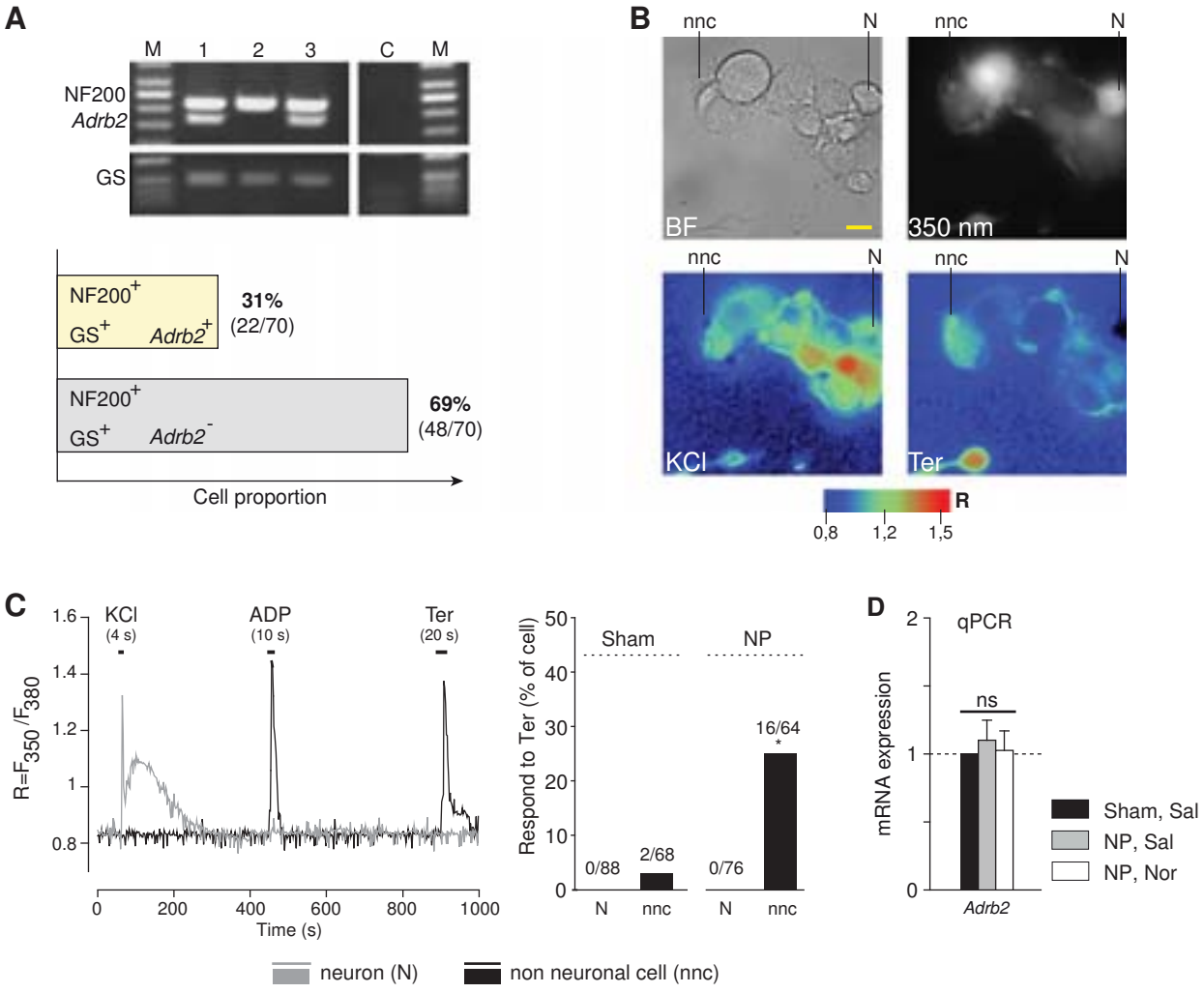

Fig. 3. β2-Adrenoceptors are on dorsal root ganglia non-neuronal cells. (A) Single cell PCR analysis for NF200, β_2-AR (*Adrb2*) and GS mRNAs (M, molecular weight ladder; 1–3, examples of 3 cells; C, negative control). (B) Calcium imaging on dissociated dorsal root ganglia cell culture, example of a typical field in bright field (BF), at 350 nm fluorescence, after 4 second high KCl application, and after 20 second terbutaline (Ter) (N, neuron; nnc, non-neuronal cell; scale bar: 20 μm). (C) Example of typical traces of calcium transients, represented as ratiometric change in fluorescence emission during bath application of high KCl (50 mM), ADP (50 μM) or the β2-AR agonist terbutaline (100 μM). Response to terbutaline was only observed in non-neuronal cells, and in a higher proportion of cells in dorsal root ganglia culture from cuff mice (*$p < 0.002$ *vs.* Sham-nnc). (D) β2-AR mRNA expression in dorsal root ganglia, evaluated by quantitative real-time PCR ($n = 3 \times 3$ per group). (Single cell PCR data are pooled from 8 independent experiments. Calcium imaging data are pooled from 6 independent experiments. Real-time PCR data represent 3 independent experiments, each of them pooling samples from 3 animals per group. ns, not significant.)

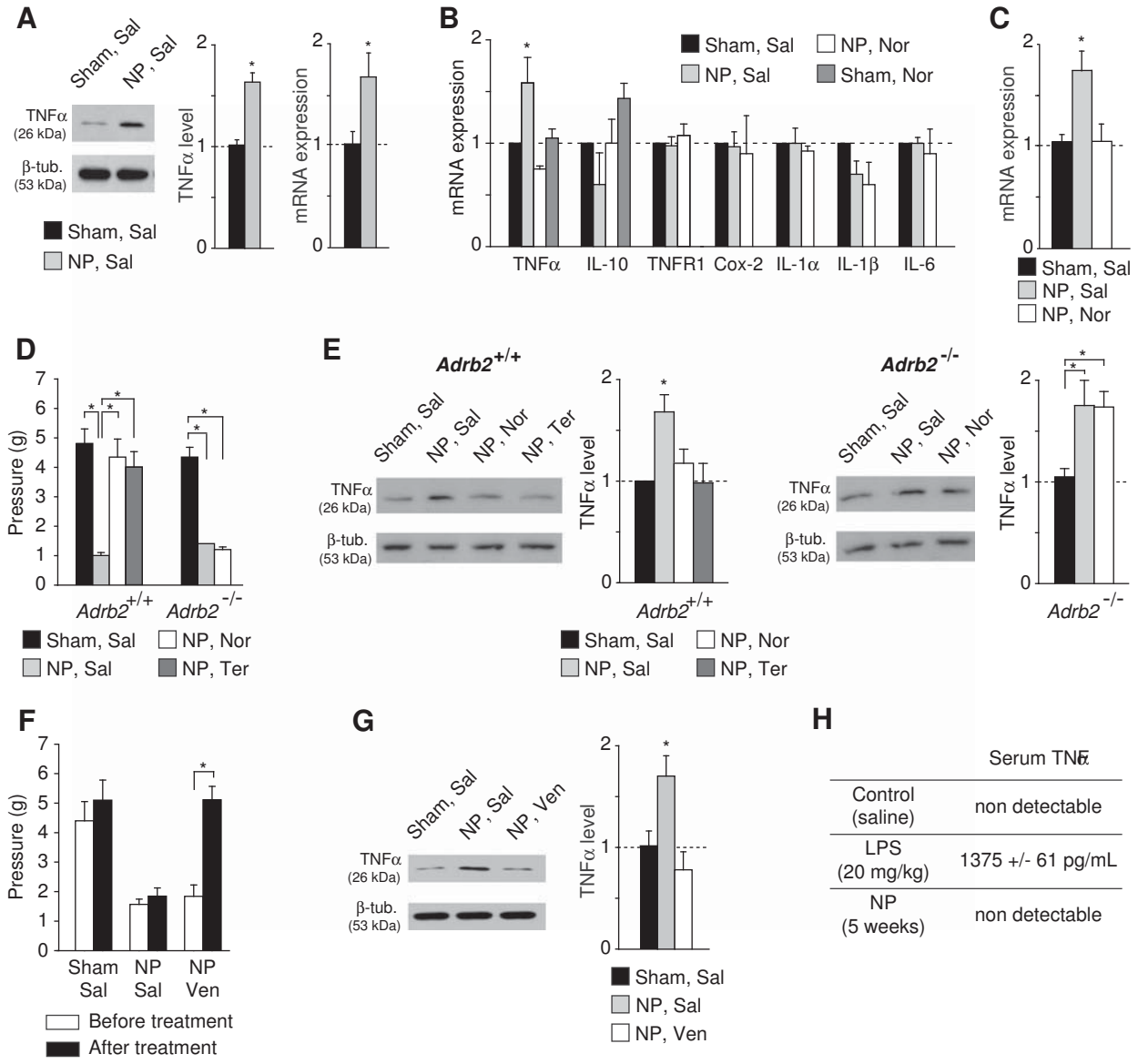

Fig. 4. Antidepressant drugs display anti-TNFα action. (A) Western blot and mRNA expression analysis on dorsal root ganglia, showing increased TNFα levels in C57BL/6J mice at 5–6 weeks after induction of the neuropathy (Western blot: $n = 12$ per group; mRNA: $n = 6$ per group; $^*p < 0.05$). (B) mRNA expression of different neuroinflammatory-related genes in dorsal root ganglia, evaluated by real-time PCR ($n = 3 \times 3$ per group, $^*p < 0.05$). (C) mRNA expression analysis confirming that the TCA nortriptyline suppressed the neuropathy-induced overexpression of TNFα mRNA ($n = 7$–9 per group, $^*p < 0.05$). (D) Behavioral analysis of neuropathic allodynia treatment in *Adrb2*$^{+/+}$ and *Adrb2*$^{-/-}$ mice. Twenty days of nortriptyline or terbutaline treatment suppressed the allodynia in wild-type mice, but nortriptyline was ineffective in *Adrb2*$^{-/-}$ mice ($n = 5$–6 per group, $^*p < 0.01$ *vs.* Sham). (E) Western blot analysis of TNFα levels in the dorsal root ganglia of *Adrb2*$^{+/+}$ and *Adrb2*$^{-/-}$ mice. Nortriptyline or terbutaline treatment suppressed the overexpression of mTNFα in wild-type mice, but nortriptyline was ineffective in *Adrb2*$^{-/-}$ mice ($n = 4$ per group, $^*p < 0.05$ *vs.* Sham-Sal). (F) Effect of a 2-week treatment with the serotonin and noradrenaline reuptake inhibitor venlafaxine on neuropathic allodynia ($n = 10$ per group, $^*p < 0.01$ *vs.* pre-treatment). (G) Western blot analysis showing the anti-TNFα action of the long-term venlafaxine treatment ($n = 8$ per group, $^*p < 0.05$ *vs.* Sham-Sal). (H) ELISA analysis of serum TNFα, LPS injection was used as positive control ($n = 6$ per group).

whereas mRNA expression of TNFα receptor 1 (TNFR1) was not affected by the neuropathy (Fig. 4B). No significant change was observed for the other inflammatory related genes Cox-2, IL-1α, IL-1β or IL-6, and a trend to a decrease was observed for the anti-inflammatory cytokine IL-10 (Fig. 4B).

The treatment with nortriptyline suppressed the mRNA overexpression of TNFα associated with the long-term neuropathy (Fig. 4B). This effect was confirmed at the mRNA level in an independent experiment in C57BL/6J mice (Fig. 4C) ($H_{2,25} = 9.11$, $p < 0.01$), and it was also observed at the protein level (Fig. 4E), using dorsal root ganglia from wild type (*Adrb2*$^{+/+}$) and β2-AR deficient (*Adrb2*$^{-/-}$) mice ($H_{5,24} = 15.46$, $p < 0.01$). Indeed, in wild type animals, the membrane-bound form of TNFα (mTNFα) increased in neuropathic mice ($p < 0.05$ against Sham-Saline), and this increase was reversed by either nortriptyline or terbutaline treatments (Fig. 4E). In *Adrb2*$^{-/-}$ mice, the antidepressant treatment was ineffective to suppress the neuropathy-induced increase in mTNFα (Fig. 4E) ($p < 0.05$ against Sham-Saline), which is in agreement with its lack of antiallodynic action in these mice (Yalcin et al., 2009a; Fig. 4D) (*Adrb2*$^{-/-}$ mice: $F_{2,15} = 77.5$, $p < 0.0001$; post-hoc: (NP-Nortriptyline = NP-Saline) < Sham-Saline at $p < 0.001$). β2-ARs are thus indirectly mediating both the molecular and the behavioral actions of nortriptyline.

To test whether this action on TNFα may be generalized to other antidepressants, we tested the selective serotonin and noradrenaline reuptake inhibitor venlafaxine. Two-week venlafaxine treatment suppressed both the neuropathic allodynia (Fig. 4F) ($F_{2,26} = 5.63$, $p < 0.01$; post-hoc: (NP-Ven = Sham-Saline) > NP-Saline at $p < 0.001$) and the neuropathic overexpression of mTNFα in the lumbar dorsal root ganglia (Fig. 4G) ($H_{2,24} = 10.64$, $p < 0.005$; (Sham-Saline = NP-Ven) < NP-Saline at $p < 0.05$).

The Western blot approach allows differentiating mTNFα (26 kDa) from soluble TNFα (17 kDa). While mTNFα changed with neuropathic pain and its treatments, the soluble form of TNFα remained undetectable in the dorsal root ganglia. We also assessed soluble TNFα in serum by ELISA, and no soluble TNFα could be detected (Fig. 4H). These results support a preferential role of mTNFα in neuropathic pain (Zhou et al., 2010), and reveal mTNFα as a downstream target for antidepressant drug action.

Anti-TNFα relief of neuropathic allodynia

The concomitant loss of anti-TNFα and antiallodynic actions of nortriptyline in *Adrb2*$^{-/-}$ mice, suggested that mTNFα could be critical for both the pathophysiology and the treatment of neuropathic pain. Thus, we tested the influence of etanercept and infliximab – clinically used anti-TNFα drugs that do not cross the blood–brain barrier – on neuropathic allodynia. Both drugs suppressed this symptom, with infliximab displaying the fastest onset of therapeutic action and maintaining its antiallodynic action over 3 weeks following a single administration (Fig. 5A) (Infliximab: $F_{24,192} = 7.68$, $p < 0.0001$; post-hoc: NP-Infliximab > NP-Saline at $p < 0.001$ on post-surgery days 16 to 35. Etanercept: $F_{24,192} = 7.34$, $p < 0.0001$; post-hoc: NP-Etanercept > NP-Saline at $p < 0.001$ on post-surgery days 18 to 35, and < Sham-Saline until post-surgery day 24). This difference between infliximab and etanercept may be related not only to differences in their half-lives (8 to 9.5 days *versus* 70 h in humans; notice instructions), but also to the fact that infliximab binds to mTNFα with higher avidity and stability than etanercept (Scallon et al., 2002). Together, the results support a critical role of peripheral mTNFα in the maintenance of neuropathic allodynia. Moreover, the therapeutic efficacy of etanercept was still observed in *Adrb2*$^{-/-}$ mice (Fig. 5B), supporting the idea that mTNFα acts downstream of these adrenoceptors (Etanercept, *Adrb2*$^{+/+}$ mice: $F_{3,20} = 14.80$, $p < 0.0001$; post-hoc: NP-Etanercept > NP-Saline at $p < 0.001$. Etanercept, *Adrb2*$^{-/-}$ mice: $F_{3,20} = 30.22$, $p < 0.0001$; post-hoc: NP-Etanercept > NP-Saline at $p < 0.001$).

Satellite cells are the source of TNFα in dorsal root ganglia

Infliximab is an artificial mouse/human monoclonal antibody directed against TNFα, and we observed that it was effective *in vivo* to suppress neuropathic allodynia. We thus perfused neuropathic mice after *in vivo* administration of infliximab, and detected this antibody to visualize its binding to mTNFα. The presence of infliximab in dorsal root ganglia after intraperitoneal injection (Fig. 6A) and its absence within the spinal cord and the brain (Figs. 6B, C) confirmed that this drug does not cross the blood–brain barrier and that its antiallodynic action is peripherally mediated. Co-staining with markers of satellite cells (GS, Fig. 7A) or neuronal cells (NT, Fig. 7A) (CGRP, NF200, SP, IB4, Fig. 7B) revealed that mTNFα was more selectively expressed by the satellite cells in the dorsal root ganglia.

Discussion

The need to improve neuropathic pain therapies is important with respect to the side effects and to the relative efficacy of existing treatments. In this context, decrypting the mechanism of existing treatments may help in improving them or in discovering new therapeutic targets. Our data show that the antiallodynic action of chronic antidepressant treatment involves the peripheral nervous system. We provide evidence that β2-ARs present on dorsal root ganglia satellite cells are responsible for this action, and that local mTNFα expression can be important for the maintenance and treatment of neuropathic pain.

Noradrenaline is a major actor in the action of antidepressant treatment (Barrot et al., 2009, 2010). Previous studies in murine models of peripheral neuropathy showed that this therapeutic effect can be blocked by lumbar intrathecal manipulations (Suzuki et al., 2008; Yalcin et al., 2009b), which supports a common hypothesis implying the recruitment of noradrenergic descending pathways in the analgesic action of antidepressants (Millan, 2002; Yoshimura and Furue, 2006). However, intrathecal manipulations also affect the closest nearby dorsal root ganglia, preventing to distinguish between central and peripheral action. By comparing noradrenergic lesions at different levels of the nervous system, we show that the peripheral noradrenergic system is necessary for the antiallodynic property of nortriptyline. In this case the local source of noradrenaline would be provided by sympathetic fibers sprouting in dorsal root ganglia that accompanies peripheral nerve injury (McLachlan et al., 1993; Ramer and Bisby, 1998). Conversely, the antiallodynic action of the antidepressant is still present after destruction of noradrenergic descending pathways. While these descending pathways are well known to exert an inhibitory control over nociceptive information (Millan, 2002), our data show that they do not play a critical role in antidepressant action. Nevertheless, the peripheral mechanism for the relief of peripheral neuropathic pain does exclude that the central component of antidepressants also participates to their therapeutic action, particularly concerning anxiodepressive and cognitive co-morbidities that often accompanies neuropathic pain (Attal et al., 2011). Indeed, neuropathic pain does alter supraspinal noradrenergic systems (Alba-Delgado et al., 2013).

Dorsal root ganglia are composed of pseudo-unipolar type neurons whose afferent axons relay sensory information into the central nervous system, and of non-neuronal cells such as resident immune cells and satellite glial cells around the soma of neurons (Hanani, 2005; von Hehn et al., 2012). β2-AR mRNA was previously detected in dorsal root ganglia (Maruo et al., 2006). In our study, we found that these receptors are more precisely expressed in non-neuronal cells, which

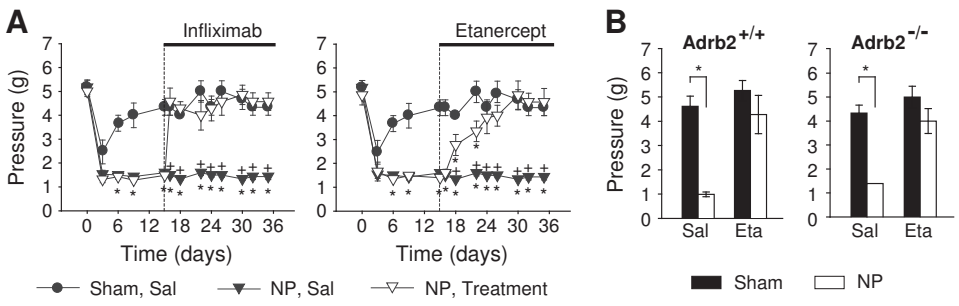

Fig. 5. Direct anti-TNFα treatments relieve neuropathic allodynia. (A) The direct anti-TNFα treatments with infliximab (10 mg/kg, injected once on day 15) or etanercept (2 mg/kg, every other day from day 15 onwards) relieved neuropathic allodynia ($n = 6$–7 per group, $^{*}p < 0.05$ *vs.* Sham, $^{+}p < 0.05$ NP-treated *vs.* NP-Sal). (B) Etanercept effect in *Adrb2*$^{+/+}$ and *Adrb2*$^{-/-}$ mice. Etanercept (Eta, 5 mg/kg, once a day for 3 weeks, from day 15 onwards) was still effective in *Adrb2*$^{-/-}$ mice ($n = 6$ per group, $^{*}p < 0.001$).

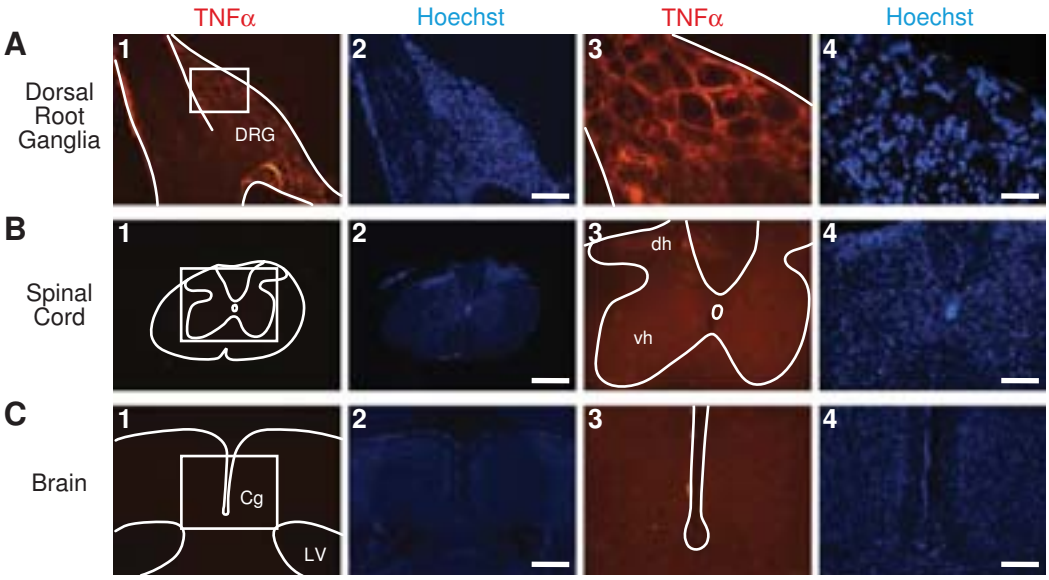

Fig. 6. Detection of TNFα by peripheral injection of infliximab. Cuff mice received acute infliximab (10 mg/kg, i.p., $n = 3$), an artificial mouse/human monoclonal antibody directed against TNFα, and were perfused 6 h post-injection. Pictures numbered 1 and 3 show TNFα detection (in red), and columns 2 and 4 show the Hoechst 33342 staining of nuclei of the same sections. Columns 3 and 4 show a larger view of the boxed areas in column 1 pictures. (A) A reticular TNFα staining was detected in the dorsal root ganglia after peripheral administration of infliximab. No staining was detected in the spinal cord (B) or brain (C) of the same animals, confirming that infliximab does not cross the blood–brain barrier. Scale bars: 500 μm in $B_{1,2}$ and $C_{1,2}$, 200 μm in $A_{1,2}$, $B_{3,4}$ and $C_{3,4}$, and 50 μm in $A_{3,4}$. Abbreviations: Cg, cingulate cortex; dh, dorsal horn; DRG, dorsal root ganglion; LV, lateral ventricle; vh, ventral horn.

are likely satellite cells expressing mTNFα. These satellite cells are the peripheral analogs of the central nervous system's astrocytes which are also known to express β2-AR on their membrane in various species including human (Mantyh et al., 1995; Trimmer et al., 1984). Moreover, we observed that the efficacy of β2-AR coupling to a transduction pathway increasing intracellular calcium is enhanced in the lumbar dorsal root ganglia under neuropathic condition. Together, our data indicate that the peripheral nerve injury does create the substrate – *i.e.* noradrenergic sprouting and enhanced β2-AR functionality – through which the antidepressant treatment acts to relieve neuropathic allodynia.

Recent data indicate that neuropathic pain may partly be considered as a neuro-immune disorder, with a critical involvement of glial and/or immune cells following nerve injury (Austin and Moalem-Taylor, 2011; Thacker et al., 2007; Vallejo et al., 2010). The activation of these cells at

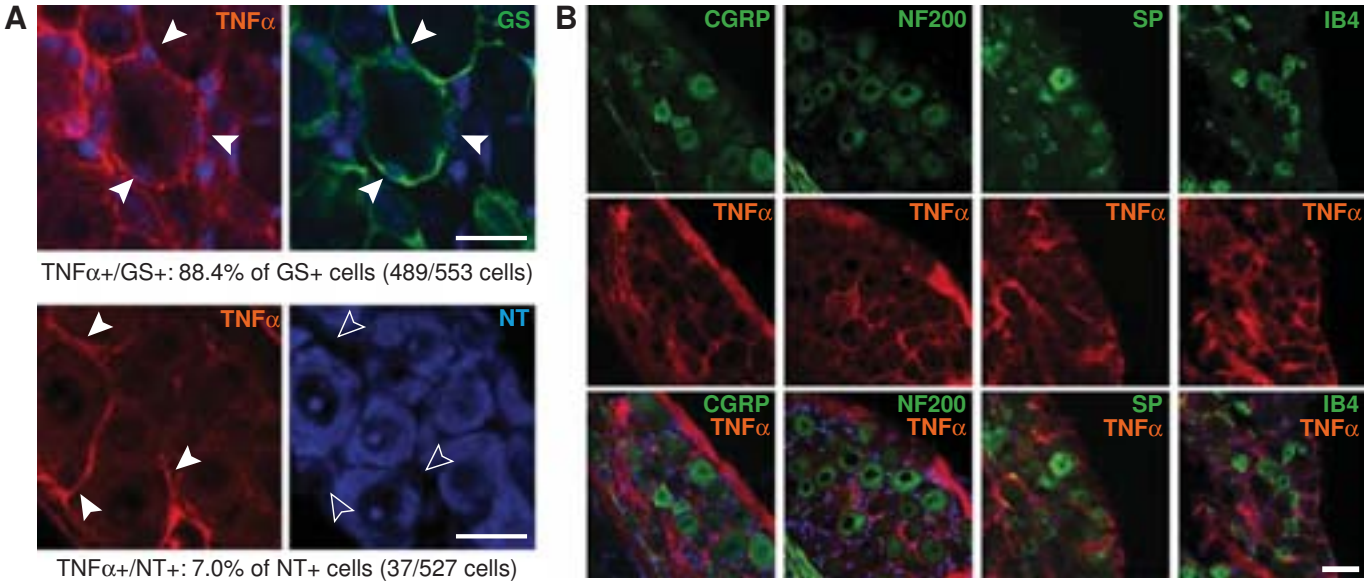

Fig. 7. Detection of TNFα in the dorsal root ganglia. Neuropathic mice received acute infliximab (10 mg/kg, i.p., $n = 3$), an artificial mouse/human monoclonal antibody directed against TNFα, and were perfused 6 h post-injection. (A) Confocal microscopy allowed to visualize TNFα (in red), cell nuclei (Hoechst 33342 in blue) and the satellite cell marker GS (in green). TNFα was detected in GS-positive satellite cells (top pictures, white arrows) in dorsal root ganglia. TNFα was rarely detected in neurons (NT in blue, bottom pictures, NeuroTrace™). Arrows on the confocal images show examples of non-neuronal TNFα staining. For both GS and NT staining, a quantification of TNFα-positive cells was done on 9 sections (3 sections × 3 animals). Scale bars: 50 μm. (B) Neuronal staining and detection of TNFα in the dorsal root ganglia. Double-staining for infliximab detection and neuronal markers was done on DRG from cuff mice ($n = 3$). The first line shows the neuronal staining, the second line shows the TNFα detection, and the third line is a merged picture. Calcitonin gene related peptide (CGRP), neurofilament 200 (NF200), substance P (SP) and the isolectin IB_4 (IB4) were used as markers of different neuronal cell populations. TNFα was not expressed by neurons, but the TNFα staining in the dorsal root ganglia formed a reticular network surrounding the neurons. Scale bar: 20 μm.

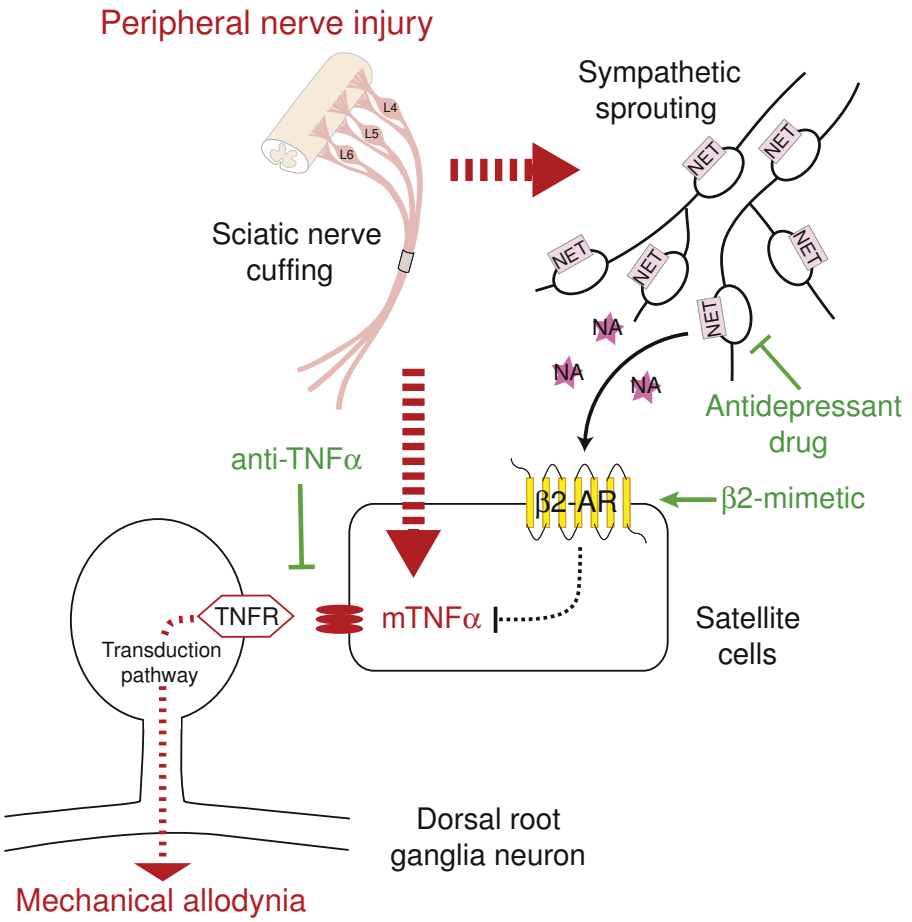

Fig. 8. Schematic of the proposed peripheral action of antidepressant drugs in the context of a peripheral neuropathy. Following sciatic nerve injury, a noradrenergic sprouting is observed in dorsal root ganglia. Antidepressant drugs inhibit noradrenaline (NA) reuptake in sympathetic varicosities (NET: transporter), thus locally increasing noradrenaline levels. Noradrenaline stimulates β2-AR expressed by non-neuronal satellite cells, which lead to decreased mTNFα production.

the lesion site or in the dorsal root ganglia for the peripheral nervous system, or in the spinal cord or supraspinal structures for the central nervous system, results in the production of cytokines (Austin and Moalem-Taylor, 2011; White et al., 2009). Pro-inflammatory cytokines produced in the peripheral nervous system after injury participate in the pathophysiology of neuropathic pain (Leung and Cahill, 2010; Sommer et al., 2001a; Wagner and Myers, 1996), particularly in its initiation. In installed neuropathic pain, some cytokines such as TNFα still display enhanced expression, which has pathophysiological relevance. Indeed, blocking TNFα can relieve neuropathic pain symptoms both in animals ((Sommer et al., 2001a, 2001b); present results) and in humans (Korhonen et al., 2005; Tobinick and Davoodifar, 2004). Our results suggest that antidepressant drugs would act as indirect anti-TNFα drugs to relieve neuropathic allodynia. This anti-TNFα effect of antidepressants is partial as it suppresses neuropathy-induced TNFα overexpression without affecting basal expression of the cytokine. The mechanism is β2-AR mediated, as indicated by the loss of both anti-TNFα and behavioral actions of nortriptyline in *Adrb2*$^{-/-}$ mice.

TNFα is primarily membrane-bound (mTNFα, 26 kDa), and this mTNFα is cleaved by the TNFα converting enzyme TACE to release the soluble peptide (sTNFα, 17 kDa). The diffusible sTNFα is the most studied form of TNFα. However, mTNFα is also biologically active as self-assembling non-covalent bound trimers (MacEwan, 2002). After chronic neuropathy, we did not detect sTNFα in the serum or in the dorsal root ganglia, but we found a significant increase of mTNFα within the dorsal root ganglia. While we cannot exclude the presence of concentrations of sTNFα that would be below detection threshold, our data however support a preferential role for mTNFα. This preferential recruitment of mTNFα in neuropathic pain has also been observed by other groups in the spinal cord (Hao et al., 2007; Peng et al., 2006), where it is produced by glial cells (Zhou et al., 2010). In the dorsal root ganglia, we observed that the mTNFα was produced by satellite cells, which might participate in neuropathic allodynia by influencing the nearby nociceptors through a local cell–cell signaling communication (Cabal-Hierro and Lazo, 2012; McCoy and Tansey, 2008; Santello and Volterra, 2012). Moreover, this local mTNFα recruitment by the neuropathic condition should not be considered as a classical inflammatory cascade. Indeed, neither IL-1 nor IL-6 expression was affected by the neuropathy at late time-points (present data), and the inhibition of the inflammatory cascade through Cox targeting by ketoprofen has no antiallodynic action (Benbouzid et al., 2008a).

The noradrenaline, at the start point of the antidepressant's therapeutic cascade, originates from sympathetic fibers sprouting in the dorsal root ganglia. Interestingly, the sympathetic nervous system has also been shown to be a potent regulator of TNFα production after lipopolysaccharide (LPS) exposure (Elenkov et al., 2000; Szelenyi et al., 2000). In LPS-induced inflammation, noradrenaline released from sympathetic nerve fibers or β-AR agonists inhibit the production of TNFα by immune and glial cells (Severn et al., 1992; van der Poll et al., 1994) and this modulatory effect may be mediated *via* β2-AR (Hetier et al., 1991; Nakamura et al., 1998). Our results support the idea that antidepressant drugs would act on neuropathic allodynia through a similar cascade of events (Fig. 8).

While a therapeutic action of anti-TNFα treatments can be observed in neuropathic pain, direct anti-TNFα therapies such as etanercept or

infliximab may not be appropriate for a large clinical use in the neuropathic pain context. Indeed, the balance of benefits/risks requires improvements as these compounds display major side effects, affecting the immune system and its ability to fight infections or cancer. Some of these risks are life-threatening with a notable incidence. Direct anti-TNFα therapy may thus be acceptable for the major inflammatory or auto-immune diseases for which these compounds are presently prescribed as second choice treatments (*i.e.* rheumatoid polyarthritis or psoriasis which is resistant to other known treatments), but they would be less acceptable for neuropathic pain which is a more widespread condition. These direct anti-TNFα treatments are fusion proteins capturing TNFα. They may be considered as too effective, blocking high pathological TNFα levels as well as physiologically relevant TNFα. Present data on the neuropathic pain models suggest that the indirect blunting of TNFα overexpression (as observed with antidepressants or β2-mimetics) could be of interest, as it may preserve physiological levels of TNFα and thus prevent some of the adverse effects of direct anti-TNFα targeting. While this hypothesis is still speculative at this point, the exploration of other partial inhibitors of TNFα production warrants further research.

In conclusion, this study highlights a novel mechanistic substrate for antidepressant drug action against neuropathic pain. By recruiting noradrenaline from sympathetic fibers sprouting in the dorsal root ganglia, antidepressant drugs stimulate local β2-ARs on non-neuronal cells. This action decreases mTNFα production and leads to the antiallodynic effect. This finding is an important step to understand the mechanism by which antidepressant drugs relieve this neurological condition. Our data obtained in a mouse model suggest that directly or indirectly targeting TNFα may potentially offer alternative therapeutics to antidepressant drugs for neuropathic pain management. It is important to note that this study was conducted in an animal model with intraperitoneal treatments. It will now require confirmation with oral administration of antidepressant drugs and a clinical validation. Such future validation will also be critical to confirm the value of this approach for translational research on neuropathic pain and its treatment.

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ÉTAT DES CONNAISSANCES ACTUELLES SUR LES MÉCANISMES D'ACTION DES ANTIDÉPRESSEURS ET DES GABAPENTINOÏDES DANS LE TRAITEMENT DE LA DOULEUR NEUROPATHIQUE

Antidepressants and gabapentinoids in neuropathic pain: mechanistic insights.

Kremer M, Salvat E, Muller A, Yalcin I and Barrot M. ***Neuroscience***, 2016, (*sous presse*).

Contrairement aux douleurs nociceptives, définies par l'International Association for the Study of Pain (IASP) comme des douleurs provoquées par l'activation des nocicepteurs avec les douleurs inflammatoires comme archétype, les douleurs neuropathiques sont peu ou pas soulagées par les antalgiques usuels comme le paracétamol ou les anti-inflammatoires non stéroïdiens. La prise en charge d'un patient souffrant de douleurs neuropathiques reste donc difficile. Les différents grades de recommandations pour le traitement des douleurs neuropathiques sont établis en fonction du niveau de preuve des études cliniques réalisées sur ces traitements (Finnerup et al., 2015). Pour autant, ces recommandations sont indépendantes des autorisations délivrées par les autorités sanitaires. Les prescripteurs tiennent donc comptent à la fois des recommandations établies par les sociétés savantes dans le domaine de la douleur et de la neurologie (Attal et al., 2006; Dworkin et al., 2007; Attal et al., 2010), mais également des autorisations de mise sur le marché obtenues pour les différentes molécules. C'est pourquoi, des antidépresseurs tels que des tricycliques (amitriptyline, Laroxyl®) ou des inhibiteurs sélectifs de la recapture de la sérotonine et de la noradrénaline (duloxétine, Cymbalta®), ainsi qu'une classe particulière d'anticonvulsivants, les gabapentinoïdes (gabapentine, Neurontin® et prégabaline, Lyrica®), sont actuellement les traitements de référence des douleurs neuropathiques.

Cette revue publiée dans *Neuroscience* fait état des connaissances actuelles sur les mécanismes d'action qui sous-tendent l'action thérapeutique des antidépresseurs et des gabapentinoïdes dans divers modèles animaux de douleurs neuropathiques. Elle se compose de trois grandes parties. La première partie, clinique, a été préparée par le Dr. Eric Salvat. Elle introduit la douleur neuropathique, sa symptomatologie, son diagnostic et ses traitements. La deuxième partie répertorie les divers composantes et mécanismes impliqués dans l'action thérapeutique des antidépresseurs lors d'un traitement prolongé. La dernière partie traite des mécanismes recrutés par les gabapentinoïdes lors du soulagement de la douleur neuropathique chez l'animal.

Pour être plus proche de la réalité clinique, où les antidépresseurs ne sont pas des analgésiques aigus mais requièrent un traitement prolongé afin d'être efficaces, différentes

stratégies d'administration prolongée d'antidépresseurs dans des modèles murin de douleur neuropathiques ont été utilisées (Benbouzid et al., 2008a; Arsenault and Sawynok, 2009; Wattiez et al., 2011). Dans le cas d'un traitement prolongé, l'hypothèse mécanistique la plus commune dans la littérature implique la noradrénaline libérée par des voies noradrénergiques issues du locus cœruleus et les récepteurs α_2-adrénergiques (α_2-AR) spinaux (Arsenault and Sawynok, 2009; Hajhashemi et al., 2014). Même si l'hypothèse d'un mécanisme spinal et / ou supraspinal est la plus étudiée, une hypothèse d'un mécanisme d'action périphérique est apparue ces dernières années. Elle suggère une action prolongée des antidépresseurs tricycliques via un recrutement des β_2-AR par la noradrénaline endogène originaire du bourgeonnement sympathique observé dans les ganglions rachidiens suite à une lésion des nerfs périphériques (Yalcin et al., 2009a; Yalcin et al., 2009b; Bohren et al., 2013 *cf. p.16)*. Le système opioïdergique, via les récepteurs mu des opioïdes (MOP), DOP et kappa des opioïdes (KOP), joue également un rôle important dans le contrôle de la douleur. Diverses études ont mis en évidence une implication de ce système dans l'action des antidépresseurs sur la douleur neuropathique, principalement par un recrutement des récepteurs MOP et / ou DOP (Marchand et al., 2003; Benbouzid et al., 2008b; Wattiez et al., 2011; Ucel et al., 2015). De nombreuses évidences montrent également une action neuroimmunitaire indirecte des antidépresseurs par une diminution de la production de cytokines pro-inflammatoires telles que le TNFα ou les interleukines (IL) 1β ou 6, anormalement augmentées dans un contexte de lésion nerveuse périphérique (Zhu et al., 2008; Bohren et al., 2013). De plus, le délai thérapeutique nécessaire à l'effet des antidépresseurs pourrait traduire la nécessité d'une plasticité moléculaire et neuronale.

Contrairement aux antidépresseurs, la gabapentine et la prégabaline peuvent avoir, en plus de leur action prolongée, une efficacité aigüe rapide à fortes doses chez l'animal (Field et al., 2006; Kremer et al., 2016). Chez l'homme, peu d'études se sont penchées sur l'existence d'un effet aigu, à court terme des gabapentinoïdes, pourtant cet effet antalgique est souvent rapporté aux praticiens par les patients et il existe dans la littérature quelques rares preuves de son existence. Par exemple, une étude clinique met en évidence qu'une unique administration de 900 mg de gabapentine aurait un effet bénéfique à court terme sur la douleur neuropathique induite par un zona (Berry and Petersen, 2005). Bien que les gabapentinoïdes soient structurellement proches du neurotransmetteur acide γ-aminobutyrique (GABA), ils ne se lient pas à ses récepteurs mais à la sous-unité α2δ des

canaux calcium dépendants du voltage (Hwang and Yaksh, 1997; Li et al., 2011; Zamponi et al., 2015), suggérant que leur action thérapeutique est médiée via une atténuation des courants calcium (Field et al., 2006; Matsuzawa et al., 2014). Ces anticonvulsivants diminuent ainsi la neurotransmission excitatrice et la sensibilisation spinale (Coderre et al., 2005; Matsumoto et al., 2006). Une action supraspinale via les contrôles descendants monoaminergiques a également été soulignée dans de nombreuses études utilisant un traitement aigu à fortes doses (Tanabe et al., 2005; Takeuchi et al., 2007a; Takeuchi et al., 2007b; Tanabe et al., 2008). Enfin, les gabapentinoïdes agissent également indirectement sur des acteurs neuroimmunitaires telles que les cytokines (Lee et al., 2013; Kremer et al., 2016).

Pour cette revue, j'ai effectué l'ensemble du travail bibliographique en amont de l'écriture, j'ai rédigé les deux grandes parties précliniques et j'ai réalisé l'ensemble des figures et tables, tout ceci sous la supervision des Drs. Ipek Yalcin et Michel Barrot.

REVIEW

ANTIDEPRESSANTS AND GABAPENTINOIDS IN NEUROPATHIC PAIN: MECHANISTIC INSIGHTS

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This article is part of a Special Issue entitled: Nociception, Pain & Analgesia.

Highlights

- Antidepressants and gabapentinoids are first line treatments for neuropathic pain.
- Chronic treatment in models of neuropathic pain provided mechanistic insights.
- Gabapentinoids target the α2δ-1 subunit of voltage-dependent calcium channels.

Abstract

Neuropathic pain arises as a consequence of a lesion or disease affecting the somatosensory system. It is generally chronic and challenging to treat. The recommended pharmacotherapy for neuropathic pain includes the use of some antidepressants, such as tricyclic antidepressants (amitriptyline…) or serotonin and noradrenaline re-uptake inhibitors (duloxetine…), and/or anticonvulsants such as the gabapentinoids gabapentin or pregabalin. Antidepressant drugs are not acute analgesics but require a chronic treatment to relieve neuropathic pain, which suggests the recruitment of secondary downstream mechanisms as well as long-term molecular and neuronal plasticity. Noradrenaline is a major actor for the action of antidepressant drugs in a neuropathic pain context. Mechanistic hypotheses have implied the recruitment of noradrenergic descending pathways as well as the peripheral recruitment of noradrenaline from sympathetic fibers sprouting into dorsal root ganglia; and importance of both α2 and β2 adrenoceptors have been reported. These monoamine re-uptake inhibitors may also indirectly act as anti-proinflammatory cytokine drugs; and their therapeutic action requires the opioid system, particularly the mu and/or delta opioid receptors. Gabapentinoids, which target the voltage-dependent calcium channels α2δ-1 subunit, inhibit calcium currents, thus decreasing the excitatory transmitter release and spinal sensitization. Gabapentinoids also activate the descending noradrenergic pain inhibitory system coupled to spinal α2 adrenoceptors. Gabapentinoid treatment may also indirectly impact on neuroimmune actors, like proinflammatory cytokines. These drugs are effective against neuropathic pain both with acute administration at high dose and with repeated administration. This review focuses on mechanistic knowledge concerning chronic antidepressant treatment and gabapentinoid treatment in a neuropathic pain context.

Abbreviations

CREB, cAMP response element-binding protein; DOP, delta opioid receptor; EFNS, European Federation of Neurological Societies; IASP, International Association for the Study of Pain; IL, interleukin; KOP, kappa opioid receptor; MOP, mu opioid receptor; NET, noradrenaline transporter; NeuPSIG, IASP Neuropathic Pain Special Interest Group; PCPA, *para*-chlorophenylalanine methyl ester; RGS, regulators of G protein signaling; SERT, serotonin transporter; SSNRI, selective serotonin and noradrenaline reuptake inhibitors; SSRI, selective serotonin reuptake inhibitors; TCA, tricyclic antidepressants; TNF-α, tumor necrosis factor α; VDCC, voltage-dependent calcium channel.

INTRODUCTION

Neuropathic pain is generally a chronic medical condition, resistant to classical analgesic drugs. Some antidepressants, together with gabapentinoids, are first-line drugs for the treatment of neuropathic pain (Attal et al., 2010). The clinical efficacy of these drugs is well documented, and preclinical research provided insights into the mechanism(s) by which these drugs are acting. This mechanistic progress was possible thanks to the development of various rodent models of neuropathic pain.

In this review, we will first provide general clinical information concerning neuropathic pain, its definition, clinical signs and symptoms and assessment, and treatments. We will then provide information, based on preclinical research in models of neuropathic pain, on the possible mechanisms of action of chronic antidepressant treatment and of gabapentinoids. It has to be noted that a large majority of the available mechanistic information about the pain relief induced by antidepressants and gabapentinoids in neuropathic pain concerns peripheral and spinal aspects.

NEUROPATHIC PAIN

From pain to neuropathic pain

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Acting as an alarm signal, acute pain triggers reactions to preserve the integrity of the organism. When pain persists beyond several months, it is considered as chronic. Such distinction only takes into account the duration, and not the underlying mechanisms. Yet, contrary to acute pain, chronic pain is regarded as an illness *per se*, accompanied by the alteration of the quality of life of the individuals, with an impact on the family, social and professional life (Maletic and Raison, 2009; Haanpaa et al., 2011; Radat et al., 2013). Pain syndromes are also distinguished according to their physiopathological mechanism, being described as either nociceptive or neuropathic. The IASP defines nociceptive pain as “pain provoked by the activation of

nociceptors", with the archetypal example of inflammatory pain. Such pain is triggered, during inflammatory episodes or tissue lesions, by the release of mediators which stimulate the nociceptive system. By contrast, neuropathic pain is defined as a "pain caused by a lesion or disease of the somatosensory system" itself (Treede et al., 2008). It reflects a pathological phenomena occurring within the nervous system, either of peripheral (nerves, plexus, roots and sensitive lymph nodes) or central (spinal cord and brain) origin. As such, neuropathic pain can be induced by a variety of pathological situations, all of them altering the physiology of the nervous system.

Clinical aspects of neuropathic pain

The emergence of neuropathic pain is conditioned by an alteration of the somatosensory pathways. The somatosensory system includes all structures allowing the perception of sensory information coming from the skin and the musculoskeletal apparatus. These information relate to the perception of mechanical, thermal or chemical stimuli. Peripheral afferents join the dorsal horn of the spinal cord through the posterior root of spinal nerves. Information is then locally processed and relayed to supraspinal levels through ascending pathways. These pathways target specific brainstem and thalamic nuclei which in turn relay the information to other cerebral areas. Conversely, descending bundles arising from cortical and subcortical areas will modulate, by inhibition or facilitation, the activity of the spinal dorsal horn networks (von Hehn et al., 2012). A lesion or a disease affecting any of these structures and/or circuits may lead to neuropathic pain. Such diversity in the topography and etiology of impairments or lesions (mechanical, infectious, toxic…) shows how complex neuropathic pain can be in its clinical expression. The diagnosis approach is presently based on expressed symptoms, signs and etiology. Thus, the pathology responsible for the alteration in the somatosensory system has to be investigated. It is customary to distinguish alterations occurring within the central nervous system itself, which mainly concerns neurological pathologies, from alterations of the peripheral nervous system, which can result from many different causes (Baron et al., 2010). In some cases, the painful neuropathic symptoms can happen after a timeframe of a few months to several years after the initial nervous lesion, particularly in the case of strokes (Andersen et al., 1995).

Signs and symptoms. The semiology of neuropathic pain associates pain and abnormal sensations and neurological deficits in a discrete nervous area (**Table 1**)

Table 1. IASP definition and assessment of sensory symptoms or signs in neuropathic pain.

Terms	Definitions
Pain	An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage
Allodynia	Pain due to a stimulus that does not normally provoke pain
Analgesia	Absence of pain in response to stimulation which would normally be painful
Dysesthesia	An unpleasant abnormal sensation, whether spontaneous or evoked
Hyperalgesia	Increased pain from a stimulus that normally provokes pain
Hyperpathia	A painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold
Hypoalgesia	Diminished pain in response to a normally painful stimulus
Neuropathic pain	Pain caused by a lesion or disease of the somatosensory nervous system
Nociception	The neural process of encoding noxious stimuli
Nociceptive pain	Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors
Nociceptive stimulus	An actually or potentially tissue-damaging event transduced and encoded by nociceptors
Nociceptor	A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli
Pain threshold	The minimum intensity of a stimulus that is perceived as painful
Paraesthesia	An abnormal sensation, whether spontaneous or evoked
Sensitization	Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs

(Bouhassira et al., 2005; Bennett et al., 2007). None of these signs and symptoms, when considered in isolation, is by itself pathognomonic, but they are all related to some extent and constitute the core of the neuropathic semiology (Attal et al., 2008). Pain and abnormal sensations associated with neuropathic pain include: *1) Paroxysmal and/or permanent spontaneous pain.* Spontaneous paroxysmal pain generally occurs by series, repeated several times a day, and are described as searing pain, resembling electrical shocks or stabbing. Permanent spontaneous pain can be felt as a painful background of compression, of burning or coldness sensation, of "pins and needles" sensations or of itching. *2) Evoked pain.* Allodynia is defined as pain due to a stimulus that does not normally provoke pain. According to the nature of the stimulus, different types of allodynia are distinguished: thermal allodynia to cold or warm, dynamic mechanical allodynia (provoked by friction) or static mechanical allodynia (provoked by a slight pressure). Hyperalgesia corresponds to increased pain from a stimulus that normally provokes pain. Hyperpathia is a painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold. *3) Abnormal sensations.* Paresthesia and dysesthesia are abnormal sensations which can happen spontaneously or be provoked by various stimuli. They are most often felt as a stinging or a tingling sensation. Dysesthesia is perceived as annoying and unpleasant. Sensation of numbness or of having the skin of cardboard quality is part of this category of symptoms. *4) Sensory deficits.* During the clinical examination, a sensitive deficit can also be discovered. It is often partial, and most of the time it affects the evoked thermal and nociceptive sensibilities. However, it can also concern the fine tactile sensitivity. This hypoesthesia can sometimes be subtle and hardly detectable; whereas in other cases a complete anesthesia of the nervous territory can be observed, as in *anesthesia dolorosa* which refers to pain felt in an area which is completely numb to touch (**Table 1**).

Diagnosis. Although the diagnosis of neuropathic pain is based on the association of signs of sensory deficits and of pain in the same neurological area, it is also established through the onset context of this pain. The diagnosis is made easier in a situation where the neurological pathology is clearly identified, in contrast with a non-neurological clinical context, where there is a risk to miss the neuropathic component of the pain described by the patient. Several screening tools for the diagnosis of neuropathic pain have been developed and validated (Bennett, 2001; Krause and Backonja, 2003; Bouhassira et al., 2005; Haanpaa et al.,

2011). In the Leeds assessment of neuropathic symptoms and signs (LANSS), which includes five symptom items and two clinical examination items (Bennett, 2001), a score of 12 or more (on a maximum possible score of 24) detects neuropathic pain with a 89-91% sensitivity and 80-94% specificity. The DN4 questionnaire ("Douleur Neuropathique en 4 questions") (Bouhassira et al., 2005) includes 7 symptom items and 3 clinical examination items. A total score of 4 or higher guides the diagnosis with a 82.9% sensitivity and a 89.9% specificity. Other tools, such as the neuropathic pain questionnaire (NPQ) (Krause and Backonja, 2003), the IDPain (Portenoy, 2006) or the PainDETECT (Freynhagen et al., 2006) consist on interview questions and do not require clinical examination. Besides these questionnaires, the clinical examination is often essential to confirm the diagnosis of neuropathic pain. The localization and the extent of the painful area confirm the neurological topology. In this painful area, the examination also looks for any sensory deficit (tactile, or thermal to warm or cold), and for sting or vibration sensations, when compared with a homologous healthy area (contralateral for instance). The evaluation of evoked pain includes the assessment, in the same area and comparing with a healthy zone, of dynamic mechanical allodynia using a brush, or of static mechanical allodynia with a blunt pin or a von Frey hair. Thermal allodynia is explored with warm and cold tubes or thermal rollers. A temporal summation can be explored by repeating stimulations with a blunt pin or a paintbrush, to reveal hyperpathia. Judging the respective importance of the different painful symptoms can be done with the Neuropathic Pain Symptom Inventory (NPSI) questionnaire, which is a self-questionnaire elaborated to discriminate and quantify each of the different components of neuropathic pain and their variations in response to a treatment (Bouhassira et al., 2004). While the present diagnosis of neuropathic pain is based on signs and symptoms only, complementary examinations can provide neuropathic pain markers. For instance, the skin biopsy evaluates the intra-epidermic density in nervous fibers (Collongues et al., 2009; Serra et al., 2012), whereas the laser-evoked potentials assess the functionality of fine fibers (Lingueglia, 2007), which allow identifying small-fiber neuropathies. A grading system based on such identification of the etiology has been proposed by Treede *et al.* for the diagnosis of neuropathic pain (Treede et al., 2008). It leads to a diagnosis of defined/probable/possible neuropathic pain, according to clinical criteria and to the results of complementary examinations. Four diagnosis criteria are thus suggested: *1.* pain with a neuroanatomical distribution suggesting a neurological systematization; *2.* clinical history of

a lesion or disease affecting the peripheral or central somatosensory system; *3.* confirmation of a neuroanatomical distribution of the pain by at least one paraclinical test; *4.* confirmation of the lesion or the neurological disease in question by at least one paraclinical test. Neuropathic pain would then be considered as "defined" if all criteria are met, "probable" by the association of criteria 1, 2 and 3 or 4, and considered as "possible" with only criteria 1 and 2. However, this gradation may not be adapted to daily clinical practice and should be used when the diagnosis is uncertain.

Pharmacological treatments of neuropathic pain

The pharmacotherapy of neuropathic pain is challenging and frequently unsatisfactory. Analgesics are poorly effective or ineffective to relieve neuropathic pain, and among current analgesics only tramadol and strong opioids (morphine, oxycodone) have shown evidence to partially relieve neuropathic pain. In 2007, the IASP Neuropathic Pain Special Interest Group (NeuPSIG), an international group of neuropathic pain experts, proposed evidence-based guidelines for the pharmacological treatment of neuropathic pain (Dworkin et al., 2007). The American Pain Society, Canadian Pain Society, Finnish Pain Society, Latin American Federation of IASP Chapters and Mexican Pain Society supported these guidelines. Simultaneously, guidelines were also proposed by the European Federation of Neurological Societies (EFNS) (Attal et al., 2006). The first-line drugs recommended by NeuPSIG and EFNS included: tricyclic antidepressants (TCAs), selective serotonin-noradrenaline reuptake inhibitors (SSNRIs) and gabapentinoids. In 2010, EFNS updated the existing evidence concerning pharmacological treatments of neuropathic pain (Attal et al., 2010), and in 2015 NeuPSIG also updated their evidence-based guidelines (Finnerup et al., 2015). TCAs, gabapentinoids and the SSNRI duloxetine remain the most recommended drugs to treat various conditions of neuropathic pain according to these guidelines.

TCAs. Efficacy of TCAs to relieve neuropathic pain first emerged from observations in depressed patients treated with imipramine (Paoli et al., 1960). Since the 1980's, clinical trials have confirmed the benefit of amitriptyline to relieve postherpetic neuropathic pain (Watson et al., 1982) and painful diabetic polyneuropathy (Max et al., 1987). This effect occurs independently from the presence of a depressive syndrome (Mico et al., 2006; Perahia et al., 2006). Moreover, the relief of neuropathic pain may also be observed without modification of the depression scores (Watson et al., 1992), and at lower doses than

necessary to treat major depression. Efficacy of systemic antidepressants is partly independent from the etiology of neuropathic pain (Saarto and Wiffen, 2007). Among TCAs, amitriptyline (25-150 mg/day) is clinically the most studied and prescribed, with demonstrated efficacy in several neuropathic pain conditions, including diabetic neuropathy, postherpetic neuralgia and central pain (Saarto and Wiffen, 2007). It alleviates specific components of neuropathic pain, such as constant pain, shooting pain and mechanical allodynia (Watson et al., 1992). Unlike classical analgesics, the effect of antidepressants on neuropathic pain is observed after prolonged treatment (Sindrup et al., 2005), suggesting the involvement of neuronal plasticity. Overall for TCAs, the number of patients needed to treat to obtain at least moderate pain relief in one patient, called Number-Needed-to-Treat (NNT), is 3.6 (95% CI 3-4.5) with no evidence of a dose-response effect (Saarto and Wiffen, 2007). Number-Needed-to-Harm (NNH) for TCAs, calculated for symptoms such as drowsiness, dizziness, dry mouth, constipation, nausea, urinary retention, sweating, headache, blurred vision, palpitations, irritability and ataxia is 6 (95% CI 4.2-10.7). These adverse effects can lead to treatment withdrawal in 20% of patients, including in clinical trials (Saarto and Wiffen, 2007). TCAs are also contraindicated in patients with cardiac troubles, glaucoma and dysuria, due to their anticholinergic properties (Roose, 2000).

Local TCAs. Similarly to some other aminergic reuptake inhibitors, TCAs can act on voltage-gated sodium channels (Barber et al., 1991), which gives TCAs antiarrhythmic properties and may also explain some cardiotoxic adverse effects at high doses (Nattel, 1985). This property of TCAs is shared by local anesthetics, and amitriptyline indeed displays an anesthetic action when delivered on a rat sciatic nerve (Gerner et al., 2001) or intrathecally (Gerner et al., 2003). This action is also present with other TCAs (Sudoh et al., 2003), even though it appears to be stronger with amitriptyline and imipramine than with nortriptyline and desipramine (Chen et al., 2004), and it has been proposed to be longer lasting than the one of the local anesthetic bupivacaine (Gerner et al., 2002; Sudoh et al., 2003). However, the interest of TCAs as local anesthetics is limited by a neurotoxicity reported by some studies. Indeed, an application of amitriptyline on a rat sciatic nerve can dose-dependently provoke peripheral axonal damage and Wallerian degeneration (Estebe and Myers, 2004; Fukushima et al., 2009), and intrathecal delivery can lead to irreversible lesions, suggesting a narrow therapeutic window (Sudoh et al., 2004). A detergent-like effect on membranes has been proposed to participate to this toxicity (Kitagawa et al., 2006). In

humans, a local application of amitriptyline induces cutaneous analgesia, which is however accompanied by redness, itching and burning sensations in the healthy subject (Gerner et al., 2003; Ho et al., 2008). In neuropathic pain patients, the application of 2 or 5% amitriptyline induces redness and isn't much effective than placebo (Lynch et al., 2005; Ho et al., 2008). Together, these data are not presently supportive of a clinical interest in using topical or intrathecal TCAs in the context of neuropathic pain. Mechanistically, however, it is still unclear whether the TCAs action on sodium channels could actively contribute to their therapeutic action at clinical doses.

SSNRIs. SSNRIs such as duloxetine (60-120 mg/day) or venlafaxine (150-225 mg/day) have been studied in many clinical trials and shown to relieve neuropathic pain. While TCA action was established on various peripheral or central neuropathic pain conditions, the clinical effects of venlafaxine and duloxetine were mainly studied on painful polyneuropathy (Attal et al., 2010; Baron et al., 2010; Finnerup et al., 2010). In addition, duloxetine has been specifically labeled for the treatment of painful diabetic polyneuropathy by the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). EFNS also recommend duloxetine and venlafaxine as first-line drugs for painful diabetic polyneuropathy; and NeuPSIG guidelines recommend duloxetine as a first-line treatment of neuropathic pain conditions, similarly to gabapentinoids and TCAs. The NNT for SSNRIs has globally been estimated to be 6.4 (95% CI 5.2–8.4), and NNH 11.8 (95% CI 9.5–15.2) (Finnerup et al., 2015). Reported adverse effects for SSNRIs include nausea, dry mouth, headache, decreased libido, dizziness, somnolence or insomnia, reduced appetite, hypertension… Venlafaxine is contraindicated in concomitant use of monamine inhibitors and in patients with uncontrolled hypertension; while duloxetine is contraindicated in hepatic and renal impairment, and should not be used in combination with CYP1A2 inhibitors. Although SSNRIs are safer than TCAs, discontinuation rates of 15–20% are still observed due to adverse effects (Goldstein et al., 2005; Gahimer et al., 2007).

Other antidepressants. Selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, paroxetine or citalopram, are better tolerated and safer to use than TCAs due to the lack of anticholinergic, antihistaminic, adrenergic and cardiac adverse effects. However, the action of SSRIs in pain conditions is controversial and there is a lack of evidence for a benefit in the treatment of neuropathic pain (Sindrup et al., 2005). For example, only small effect of paroxetine and citalopram, or no effect of fluoxetine, has been reported in painful

diabetic neuropathy (Sindrup et al., 1990; Max et al., 1992; Sindrup et al., 1992; Sindrup et al., 2005). The atypical antidepressant bupropion, which inhibits noradrenaline and dopamine reuptake, has been reported in one study to be potentially effective against neuropathic pain (Semenchuk et al., 2001).

Gabapentinoids. The gabapentinoids gabapentin and pregabalin are both ligands of the $\alpha_2\delta$ subunit of voltage-dependent calcium channels. They globally have a favorable adverse effect profile, most frequent adverse effects including dizziness, somnolence, peripheral edema, weight gain, asthenia, headache and dry mouth (Dworkin et al., 2007). Gabapentin was first developed to be an anticonvulsant agent but it is now approved by the FDA for the management of post-herpetic neuropathic pain. NNT is 6.3 (95% CI 5.0–8.3) for gabapentin and there is no evidence of a dose-response effect. Gabapentin is generally safe and its NNH is 25.6 (95% CI 15.3–78.6). It has no clinically important drug interaction, and the main dose-limiting side effects are somnolence and dizziness. In elderly patients, gabapentin can however cause or exacerbate cognitive impairments. The effective dose is usually between 1200 and 3600 mg/day in three doses, gradually reached over several weeks. The onset of analgesic activity occurs with a delay after therapeutic dose is achieved. Similar to gabapentin, pregabalin is FDA approved for the management of post-herpetic or diabetic neuropathic pain, with a well-established efficacy (Moore et al., 2009). It has similar efficacy and tolerability compared to gabapentin, but display a more pronounced dose-response effect with better responses at 600 mg daily than 300 mg. Combined NNT is 7.7 (95% CI 6.5–9.4), and NNH is 13.9 (95% CI 11.6–17.4) (Finnerup et al., 2015). Due to titration procedures, the onset of pain relief occurs sooner with pregabalin than with gabapentin. Indeed, 150 mg/day of pregabalin can already be effective (Dworkin et al., 2007). The maximum benefits typically occur after two weeks of treatment at target dose of 300–600 mg/day.

Carbamazepine. Carbamazepine acts on voltage-dependent sodium channels. It is the drug of choice in trigeminal neuralgia since the 1960's (Blom, 1962; Sindrup and Jensen, 2002). A significant benefit is observed after 5 to 14 days of treatment in 89% of the patients (McQuay et al., 1995).

Opioid analgesics. Tramadol and opioids are considered as second-line drugs except in circumstances such as the treatment of acute neuropathic pain or neuropathic cancer pain, episodic exacerbation of severe pain and during the titration of a first-line medication

(Attal et al., 2006). Like oxycodone (Eisenberg et al., 2006), tramadol reduces pain in diabetic painful polyneuropathy (Hollingshead et al., 2006). Interestingly, tramadol also acts partially trough an antidepressant-like mechanism implying serotonin-noradrenaline reuptake inhibition. Adverse effects of tramadol include dizziness, nausea, constipation, somnolence and an increased risk of seizure in patients with epilepsy. There is also a risk of serotonergic syndrome if tramadol is associated with other serotoninergic drugs (e.g. antidepressants or lithium). Tapentadol, a successor of tramadol with a dual mechanism of action as mu-opioid receptor agonist and noradrenaline reuptake inhibitor, has been recently developed (Tzschentke et al., 2007). Tapentadol was approved in 2008 by the US FDA for the treatment of moderate to severe acute pain, and of severe painful diabetic polyneuropathy in patients who need around-the-clock medication to relieve pain that cannot be controlled by the use of other treatments. Recently, tapentadol has also been approved by other agencies in Australia and UK. While its mechanism of action is of interest, there is still a lack of strong published evidence for the effectiveness of tapentadol against neuropathic pain, and there are thus still inconclusive recommendations from NeuPSIG for its use in neuropathic pain conditions (Finnerup et al., 2015). Morphine and oxycodone have shown efficacy in post-herpetic neuropathic pain but the dosage may be higher than normally required for nociceptive pain. Anyway, strong opioids are now recommended as third line treatment (Attal et al., 2010; Finnerup et al., 2015), because of potential risks of misuse, abuse, overdose mortality and other opioid-related morbidity.

ANTIDEPRESSANT DRUG MECHANISMS IN NEUROPATHIC PAIN

Long-term effects of antidepressants in rodents

A notable set of scientific information regarding the pain-relieving action of antidepressants was in fact obtained using acute pain protocols in animals, for which the antinociceptive action of antidepressants was tested using various noxious stimuli (thermal, chemical or mechanical). While antidepressants at high dose may be effective in reducing nociceptive responses in different models (De Vry et al., 2004; Bomholt et al., 2005; Mico et al., 2006; Onal et al., 2007; Suzuki et al., 2008; Le Cudennec and Castagne, 2014), it does not reflect the clinical use of these drugs. Indeed, when given chronically, antidepressants are first line

Table 2. Proposed mechanisms of action for the analgesic effect of long-term antidepressants.

This articles' selection was restricted to articles with chronic or sustained antidepressant treatments and only in neuropathic pain models. *Abbreviations:* 5-HT, serotonin; ACC, anterior cingulate cortex; AR, adrenoceptor; BDNF, brain-derived neurotrophic factor; CCI, chronic constriction injury; CNS, central nervous system; CREB, cAMP response element-binding protein; d, days; DOP, delta opioid; EAAT, excitatory amino acid transporters; GABA, gamma-aminobutyric acid; i, injections every half-life; i.c.v, intracerebroventricular; IL, interleukin; i.p, intraperitoneal; KOP, kappa opioid; MOP, mu opioid; NA, noradrenaline; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NP, nucleus pulposus; NR2B, N-methyl D-aspartate receptor subtype 2B; NRI, noradrenaline reuptake inhibitors; ob/ob, obese leptin deficient mice; OXP, oxaliplatin; p.o, per os; PLCγ, phosphoinositide phospholipase γ; PSNL, partial sciatic nerve ligation; RGS, regulator of G protein signaling; s.c, subcutaneous; SNI, spared nerve injury; SNL, spinal nerve ligation; SNRI, selective noradrenaline reuptake inhibitors; SSRI, selective serotonin reuptake inhibitors; STZ, streptozotocin; TCA, tricyclic antidepressant; TNF, tumor necrosis factor; w, weeks.

Dose (mg/kg)	Time	Species	Pain models	Pain parameters	Comments	References
AMITRIPTYLINE (TCA)						
10 (i.p.)	7d	Rat	PSNL	Thermal hyperalgesia ↓ Mechanical allodynia ↔	Maintain spinal cord $GABA_B$ receptor activity; Onset: 7 days	McCarson et al. (2005)
5 (i.p.)	21d	Mice	Cuff	Mechanical allodynia ↓	Involvement of endogenous opioid system; Onset: 13 days	Benbouzid et al. (2008a)
5 (i.p.)	20d	Mice	Cuff	Mechanical allodynia ↓	Involvement of DOP receptors; Onset: 2 weeks	Benbouzid et al. (2008b)
10 (i.p.)	3-20d	Rat	CCI	Thermal hyperalgesia ↓ *at day 0 or 4 only*	Decrease TNF-α immunoreactivity in the hippocampus and injured nerve; Onset: 2 days	Sud et al. (2008)
10 (i.p.) 16 (p.o.)	7d	Rat	SNI	Chemogenic hypersensitivity ↓ Chemogenic hyposensitivity ↓ Mechanical allodynia ↔	Involvement of spinal noradrenergic systems and growth factors; No onset: test day 14	Arsenault and Sawynok (2009)
10 (i.p.)	5d	Rat	SNI	Mechanical allodynia ↓	Increase the expression of EAATs; Onset: 5 days	Mao and Yang (2010)
5, 10 (p.o.)	27d	Rat	OXP	Mechanical allodynia ↓ Cold hyperalgesia ↔	Reverse the expression of NR2B in spinal cord; Onset: 28 days for 5 and 14 days for 10 mg/kg	Sada and al. (2012)
CLOMIPRAMINE (TCA)						
5 (s.c.)	5i	Rat	CCI STZ	Mechanical hyperalgesia↓	Involvement of opioid system; Onset: 243 min for diabetic rats and 398 min for CCI	Wattiez et al. (2011)
IMIPRAMINE (TCA)						
5 to 30 (i.p.)	21d	Mice	PSNL	Tactile hypersensitivity ↓ Thermal hyperalgesia ↓	Reduce CREB and PLCγ-1 phosphorylation; Onset: 2 weeks for 30 mg/kg and 3 weeks for 5 and 10 mg/kg	Kusuda et al.(2013)
NORTRIPTYLINE (TCA)						
0.5 to 5 (i.p.)	21d	Mice	Cuff	Mechanical allodynia↓ ↔ *at dose 0.5*	Involvement of endogenous opioid system; Onset: 10 days	Benbouzid et al. (2008a)
0.5 to 5 (i.p.)	20d	Mice	Cuff	Mechanical allodynia ↓ ↔ *at dose 0.5*	Require DOP receptors; Onset: 2 weeks	Benbouzid et al. (2008b)
5 (i.p.)	3w	Mice	Cuff	Mechanical allodynia ↓	Involvement of β2-ARs; Onset: 2 weeks	Yalcin et al. (2009a)
5 (i.p.)	20d	Mice	Cuff	Mechanical allodynia ↓	MOP receptors are not critical; Onset: 10 days	Bohren et al. (2010)
5 (i.p.)	3w	Mice	Cuff	Mechanical allodynia ↓	Induce an indirect anti-TNF-α action; Onset: 2 weeks	Bohren et al. (2013)
5 (i.p.)	2 to 3w	Mice	ob/ob	Mechanical allodynia ↓	Involvement of β2-ARs; Onset: 10 days	Kusuda et al. (2013)
5 (i.p.)	20d	Mice	Cuff	Mechanical allodynia ↓	KOP receptors are not necessary; Onset: 12 days	Megat et al. (2015)
DESIPRAMINE (TCA)						
5 (i.p.)	2 to 4w	Mice	Cuff	Mechanical allodynia ↓	Involvement of β2-ARs but not α2-ARs; Onset: 2 weeks	Yalcin et al. (2009b)
10, 20 (i.p)	15d	Mice	SNI	Mechanical allodynia ↓	Modulator role of RGS-4; Onset: 6 and 9 days for 20 and 10 mg/kg respectively	Stratinaki et al. (2013)
REBOXETINE (NRI)						
0.8 (i.p.)	2 to 4w	Mice	Cuff	Mechanical allodynia ↓	Involvement of β2-ARs but not α2-ARs; Onset: 10 days	Yalcin et al. (2009b)
DULOXETINE (SNRI)						
3 (i.p.)	5i	Rat	CCI STZ	Mechanical hyperalgesia ↓	Opioid system not involved; Onset: 232 min for STZ and 232 min for CCI	Wattiez et al. (2011)

treatments against chronic forms of neuropathic pain. For this reason, we limited this review to data from studies reporting *sustained* antidepressant treatment, in animal models of neuropathic pain (**Table 2**). It is also to be noted that a large part of the available literature in a context of neuropathic pain focuses on a spinal-based hypothesis of antidepressants' mechanism.

Most studies on antidepressant action in neuropathic pain use models of nerve injury. The injury can be induced by chronic nerve compression, either by partial sciatic nerve ligation, i.e. tight ligation of one-third to half of the sciatic nerve (Seltzer et al., 1990), spinal nerve ligation, i.e. tight ligation of L5 and L6 spinal nerves (Kim and Chung, 1992), chronic constriction injury, consisting in loosely constrictive ligatures applied around the sciatic nerve (Bennett and Xie, 1988), or by implanting a cuff around the sciatic nerve (Benbouzid et al., 2008c; Yalcin et al., 2014). Other lesional models can include an axotomy, as with the spared nerve injury relying on the axotomy of two of the three branches of the sciatic nerve (Decosterd and Woolf, 2000). Allografted nucleus pulposus into epidural space (Kawakami et al., 1996) has also been used. Beside nerve lesion, diseases affecting the somatosensory system constitute another main etiology of neuropathic pain. Thus, antidepressant action has been studied in models of diabetic polyneuropathy, either genetically or chemically-induced, including the use of obese leptin deficient mice (*ob/ob*) which constitutes one of the genetic models of type 2 diabetes (Allen et al., 2004; Choucair-Jaafar et al., 2014), or the more commonly used model of streptozotocin-induced diabetic neuropathy (Lenzen, 2008). Lastly, some studies on antidepressant action were conducted in models of chemotherapy-induced neuropathic pain, using oxaliplatin (Cavaletti et al., 2001), even though antidepressant effectiveness might be clinically poor against chemotherapy-induced neuropathic pain (Hammack et al., 2002; Attal et al., 2006; Kautio et al., 2008).

Various studies have shown that long-term antidepressant treatment has an antiallodynic and/or an antihyperalgesic effect in above models of neuropathic pain (**Table 2**). This action is found regardless of the route of administration (intraperitoneal, subcutaneous or oral). In addition, these effects have been reported with various antidepressants, including TCAs such as amitriptyline, nortriptyline, imipramine, desipramine and clomipramine, or SSNRIs such as duloxetine, venlafaxine and milnacipram. Usually, the dose necessary to achieve antiallodynia and/or antihyperalgesia with sustained treatment is ineffective in inducing acute analgesia (Obata et al., 2005; Rode et al., 2006; Benbouzid et

VENLAFAXINE (SNRI)						
2.5 to 10 (s.c.)	5i	Rat	CCI	Mechanical hyperalgesia ↓	Involvement of NA and 5-HT systems but not endogenous opioid system; Onset: 30 min after the fifth injection for 10 and 45 min for 5 mg/kg	Marchand et al. (2003a)
10, 2.5 (s.c.)	5i	Rat	CCI	C-reflex wind-up ↓	Inhibit spinal wind-up at 10 mg/kg by blockade of central 5-HT_{1A} receptors	Marchand et al. (2004)
10 (i.p.)	2 to 4w	Mice	Cuff	Mechanical allodynia ↓	Involvement of β2-ARs but not α2-ARs; Onset: 10 days	Yalcin et al. (2009b)
5 (i.p.)	3w	Mice	Cuff	Mechanical allodynia ↓	Induce an indirect anti-TNF-α action; Onset: 2 weeks	Bohren et al. (2013)
10, 20 (i.p.)	14d	Rat	CCI	Thermal hyperalgesia↓ Tactile mechanical and Cold allodynia ↔	Involvement of α2-ARs; Heat hyperalgesia onset: 1 week and 10 days for 20 and 10 mg/kg respectively; Mechanical allodynia onset: 10 and 14 days for 20 and 10 mg/kg respectively	Hajhashemi et al. (2014)
MILNACIPRAN (SNRI)						
5, 10 (osmotic pump)	14d	Rat	CCI	Mechanical allodynia ↓ ↔ *at dose 5 mg/kg*	Suppress nociceptive-induced activation (Fos) of the ACC; Onset: 7 days	Takeda et al. (2009)
10, 20 (i.p.)	5i	Rat	CCI STZ	Mechanical hyperalgesia ↓	Involvement of opioid system in CCI not in STZ; Onset: 660 min for STZ and 660 min for CCI	Wattiez et al. (2011)
MIRTAZAPINE (tetracyclic antidepressant)						
10 to 30 (p.o.)	14d	Rat	SNL	Mechanical hyperalgesia ↓; ↔ *at dose 10* Thermal hyperalgesia ↓ ↔ *at dose 10*	Inhibit cerebral proinflammatory cytokines production and NFκB activation; Onset: 7 and 11 for 30 and 20 mg/kg respectively for thermal and mechanical hyperalgesia	Zhu et al. (2008)
10 to 30 (p.o.)	28d	Rat	OXP	Mechanical allodynia ↓; ↔ *at dose 10* Cold hyperalgesia ↔	Reverse the up-regulation of spinal NR2B via activating 5-HT_{1A} receptors; Onset: 14 days	Liu et al. (2013)
MIANSERIN (tetracyclic antidepressant)						
10 (i.p.)	5i	Rat	STZ	Mechanical hyperalgesia ↓ *(after the 5 injections ≈ 12 hours)*	Antihyperalgesic effect if they disrupt interactions between 5-HT_{2A} receptors and PDZ protein; Onset: acute test only	Pichon et al. (2010)
30, 45 (p.o.)	14d	Rat	STZ	Mechanical hyperalgesia↓ Thermal hyperalgesia↓ Mechanical allodynia↓ Thermal allodynia↓	Increase catecholamine levels in the synaptic cleft, Involvement of α and β-ARs and opioidergics receptors; Onset: 7 days	Uçel et al. (2015)
FLUOXETINE (SSRI)						
20 (s.c.)	21d	Rat	OXP	Mechanical pressure ↓ Mechanical allodynia ↓ Cold hyperalgesia ↓	Increase expression of 5-HT_{2C} receptor mRNA (spinal cord/amygdale) and protein levels (spinal cord); Onset: 22 days	Baptista-de-Souza et al. (2014)

al., 2008a; Saika et al., 2009; Katsuyama et al., 2013; Kusuda et al., 2013; Le Cudennec and Castagne, 2014), higher doses being indeed necessary to observe an acute pain-relieving action. Whether mechanisms of the acute and the long-term antidepressant treatments may be similar or differ is still an open question. However, the chronic aspect of antidepressant treatment is likely an important feature to consider. In the field of depression-related research, the delay in the onset of the therapeutic effect led to the idea that antidepressants act via long-term molecular and neural plasticity, which can affect chromatin regulation and gene expression, recruits neurotrophins, stimulates dendritic arborization and spines as well as neurogenesis (Vialou et al., 2013; Duman and Duman, 2015; Rantamaki and Yalcin, 2016). Although the therapeutic onset for pain is slightly faster than for depression, the effect is nevertheless seen after several days of treatment, suggesting likewise the involvement of treatment-induced molecular and neural plasticity events, which might impact on plasticity changes accompanying neuropathic pain (Costigan et al., 2009; Nardone et al., 2013; West et al., 2015).

Monoaminergic component

Noradrenaline versus serotonin

The ability of antidepressants to inhibit monoamine reuptake is generally considered as the main mechanism leading to both their thymoleptic and analgesic effects. However, all antidepressants do not target the serotonergic and the noradrenergic systems with the same efficacy; and clinical and preclinical information are supportive of a differential importance of each of these systems.

Clinical studies show that TCAs have the highest therapeutic efficacy (Attal et al., 2010; Finnerup et al., 2015), even though their use is limited by adverse effects. Among these TCAs, amitriptyline is a molecule of reference for preclinical studies (Mico et al., 2006) as its clinical use is quite widespread both in the psychiatry and in the pain fields (Mico et al., 2006; Attal et al., 2010; Finnerup et al., 2015). While amitriptyline is with 7 fold higher potency for the serotonin transporter (SERT) than for the noradrenaline transporter (NET), its demethylated active metabolite nortriptyline is 40 fold more potent on NET than on SERT. A large part of amitriptyline action on neuropathic pain may thus be related to its metabolite nortriptyline. More recently, the efficacy of the non-tricyclic antidepressants SSNRIs has also been observed in neuropathic pain models (Marchand et al., 2003a; Marchand et al., 2004;

Yalcin et al., 2009b; Wattiez et al., 2011; Hajhashemi et al., 2014; Cegielska-Perun et al., 2015; Finnerup et al., 2015). Since both TCAs and SSNRIs target the serotonergic and the noradrenergic systems, the question of the involvement of these systems in the pain-relieving effect of antidepressants was raised. A study suggested that the antinociceptive effect of repeated administrations of venlafaxine depends on both the serotonergic and the noradrenergic systems (Marchand et al., 2003a). Other studies, however, suggest that the noradrenergic system is the main player in the analgesic effect of TCAS or SSNRIs (Suzuki et al., 2008; Arsenault and Sawynok, 2009; Yalcin et al., 2009a; Yalcin et al., 2009b; Bohren et al., 2013). It may be proposed that the necessary component of antidepressant drug action on neuropathic pain is the noradrenergic one, but that the serotonergic component can modulate this action.

The importance of noradrenaline is also supported by clinical and preclinical studies using selective noradrenaline uptake inhibitors, such as reboxetine (Krell et al., 2005; Yalcin et al., 2009b; Hughes et al., 2015), even though this class of antidepressants is not widely used in clinic due to their adverse effects. On the other hand, SSRIs are widely used for the treatment of depression, with fewer side effects. However, data concerning their use in neuropathic pain are not consistent. Indeed, some clinical studies suggested an efficacy of these molecules, while most studies found them not or poorly effective (Max et al., 1992; Rowbotham et al., 2005; Sindrup et al., 2005; Otto et al., 2008). Meta-analyses taking into consideration the quality of the clinical studies, and evidence-based recommendations for treatments, are supportive of a limited action of SSRIs against neuropathic pain (Attal et al., 2006; Saarto and Wiffen, 2007; Attal et al., 2010; Dharmshaktu et al., 2012). However, most preclinical studies with long-term SSRI treatments are not concordant with clinical findings. Many studies report an action, often partial, on nociceptive parameters in neuropathic pain models (Matsuzawa-Yanagida et al., 2008; Katsuyama et al., 2013; Baptista-de-Souza et al., 2014; Zarei et al., 2014); whereas some others show a lack of effect (Jett et al., 1997; Benbouzid et al., 2008a), which is more in line with clinical recommendations. Moreover, *para*-chlorophenylalanine methyl ester (PCPA), an inhibitor of serotonin synthesis, inhibited the antinociceptive effect of repeated venlafaxine in the chronic constriction injury model (Marchand et al., 2003a), while it did not alter the long-term effect of the tetracyclic antidepressant mianserin in the streptozotocin model of diabetic polyneuropathy (Ucel et al., 2015).

While an action of SSRIs in animal models has been observed, the data regarding the involved serotonergic receptors are disparate. For instance, it has been shown that SSRIs could become more effective when spinal 5-HT_{2A} receptors are disconnected from their associated PDZ-domain containing proteins (Pichon et al., 2010). Indeed these postsynaptic PDZ proteins may sequester 5-HT_{2A} receptors to the membrane in a context of neuropathic pain, thus limiting the analgesic action of SSRIs. The action of chronic fluoxetine treatment has also been associated with increased expression of 5-HT_{2C} receptors within some components of the central nociceptive system (Baptista-de-Souza et al., 2014). Besides SSRIs, some rare studies also addressed the role of serotonergic receptors in the action of repeated TCA, SSNRI or tetracyclic antidepressant. For instance, repeated mirtazapine treatment attenuates oxaliplatin-induced mechanical allodynia in rats, and reverses the oxaliplatin-induced up-regulation of spinal NMDA receptor subunit NR2B, via activation of 5-HT_{1A} receptors (Liu et al., 2013). Furthermore, activation of 5-HT_{1A} receptors by a systemic administration of a direct agonist enhances the inhibitory effect of venlafaxine on locus coeruleus neuron activity in long-term venlafaxine-treated naive animals (Berrocoso and Mico, 2007). These results however differ from previous data showing that a selective 5-HT_{1A} receptor antagonist decreased the delay of action, and potentiated the antinociceptive effect, of a TCA in mononeuropathic and in diabetic rats (Ardid et al., 2001). In addition, in mononeuropathic rat, repeated administration of venlafaxine (five successive injections every half-life) induced a progressive reduction of spinal wind-up, and this effect is centrally potentiated by intracerebroventricular administration of a 5-HT_{1A} receptor antagonist (Marchand et al., 2004). Last, it has recently been shown that long-term administration of a TCA in naïve animals may lead to both allodynia and hyperalgesia, and these effects would be associated with an increase in the levels of pronociceptive cytokines resulting from 5-HT_3-induced activation (Mika et al., 2015).

Both the positive and the negative results regarding the serotonergic system lead to the idea that the chronic inhibition of serotonin uptake alone may not be sufficient to efficiently alleviate neuropathic-induced allodynia, which would be consistent with practice in human clinic (Finnerup et al., 2015). Antidepressant's action on the noradrenergic system thus appears important.

Involved adrenoceptors

The action of noradrenaline is mediated through three classes of receptors (α_1, α_2 and β), representing ten different subtypes. All noradrenaline receptors belong to the family of G-proteins coupled receptors; α_1 receptors being coupled with Gq proteins, α_2 receptors with Gi and β receptors with Gs proteins (Millan, 2002). Some studies addressed the role of the various adrenergic receptors in the mechanism of action of antidepressants' treatments, using various models of neuropathic pain. For instance, the analgesic effect produced by acute amitriptyline at high dose is reduced in α2A adrenoceptor deficient mice (Ozdogan et al., 2004). Similarly, an acute administration of the α2 adrenoceptor antagonist yohimbine decreased the anti-nociceptive effect of either acute or chronic administration of the SSNRI venlafaxine on heat hyperalgesia (Hajhashemi et al., 2014). However, for other authors, the antiallodynic effect of a long-term administration of TCAs or SSNRIs more specifically involves the β2 adrenoceptor, since blocking this receptor, or using mice deficient for β2 adrenoceptors, fully prevents the antiallodynic action of the antidepressants (Yalcin et al., 2009a; Yalcin et al., 2009b; Bohren et al., 2013; Choucair-Jaafar et al., 2014), while the manipulation of α2 adrenoceptors has no effect. Interestingly, a recent study on the effect of long-term mianserin treatment, in a model of diabetic neuropathic pain, proposes that the therapeutic effect depends on both α and β adrenoceptors (Ucel et al., 2015).

Neuroanatomical substrate of action

Noradrenaline can be released within supraspinal structures (El Mansari et al., 2010; Llorca-Torralba et al., 2016), at spinal level by descending noradrenergic inhibitory controls (Yoshimura and Furue, 2006; Llorca-Torralba et al., 2016), or at peripheral level in the dorsal root ganglia following neuropathy-induced noradrenergic sprouting of sympathetic nervous system (McLachlan et al., 1993; Ramer and Bisby, 1998).

Spinal site of action. Similarly to the identification of the adrenoceptor(s) responsible for antidepressants action on neuropathic pain, the identification of the source of noradrenaline required for the therapeutic action is giving conflicting results. Unfortunately, most studies looking for the potential site of action of antidepressants have considered acute treatments rather than chronic ones. Some studies using murine models of peripheral neuropathy showed that the therapeutic effect of long-term antidepressant treatment can be blocked by lumbar intrathecal injection of the neurotoxin 6-hydroxydopamine, which

depletes spinal cord noradrenaline levels (Arsenault and Sawynok, 2009), or of a β2 adrenoceptor antagonist (Yalcin et al., 2009b). This would support the hypothesis implying the recruitment of noradrenergic descending pathways in the analgesic action of antidepressants.

Supraspinal site of action. Although aminergic descending pathways are the most studied, some works also addressed the role of supraspinal structures in the therapeutic efficacy of antidepressants in the context of neuropathic pain. The onset of therapeutic action of long-term desipramine treatment in mice with spared nerve injury is for example controlled by RGS proteins, regulators of G protein signaling, in the nucleus accumbens (Mitsi et al., 2015). Indeed, *Rgs9-2* deficient mice display an accelerated onset of desipramine antiallodynic action, while viral-mediated overexpression of RGS9-2 in the nucleus accumbens blunts this antiallodynic action (Mitsi et al., 2015). Similarly, biochemical and behavioral findings support the notion that RGS4 proteins influences desipramine action on neuropathic allodynia. Indeed, in the spared nerve injury model of neuropathic pain in mice, mild doses of desipramine are less effective in *Rgs4*-deficient mice (Stratinaki et al., 2013).

Peripheral site of action. While early studies focused on a central action of antidepressants in the relief of neuropathic pain through descending pathways, some recent studies highlighted a possible peripheral mechanism. Indeed, intrathecal injections of aminergic toxins at lumbar level do also affect the nearby dorsal root ganglia (Bohren et al., 2013), which prevent differentiating between central and peripheral action. By comparing noradrenergic lesions at different levels of the nervous system, it has been evidenced that the peripheral noradrenergic system is in fact necessary to the antiallodynic property of long-term nortriptyline (Bohren et al., 2013). In this case the local source of noradrenaline would be provided by sympathetic fibers sprouting in dorsal root ganglia that accompanies peripheral nerve injury (McLachlan et al., 1993; Ramer and Bisby, 1998).

Opioidergic component

The opioid system via mu (MOP), delta (DOP) and kappa (KOP) receptors plays a crucial role in the inhibitory controls of pain (Gaveriaux-Ruff and Kieffer, 2002; Dierich and Kieffer, 2004), and different studies evidenced an involvement of the opioid system in the action of antidepressants on neuropathic pain. Indeed, the opioid receptor antagonist naloxone

blocks the effect of repeated or long-term antidepressant treatments in models of neuropathic pain (Marchand et al., 2003b; Benbouzid et al., 2008a; Wattiez et al., 2011; Ucel et al., 2015). However, such influence is not always observed. In particular, it has been noted that naloxone only blocks antidepressants' action in repeated treatment conditions that do not also display analgesia in non-painful animals (Marchand et al., 2003a; Wattiez et al., 2011).

Both the identity and the location of the opioid receptors implicated in antidepressants' action have been studied. It has been suggested that the antiallodynic action of repeated clomipramine requires the activation of MOP receptors in the spinal cord and of DOP receptors at supraspinal levels (Marchand et al., 2003b). However, more recent research using transgenic mice deficient for opioid receptors showed a preferential role of DOP receptors in the antiallodynic action of long-term TCA treatment (Benbouzid et al., 2008b; Choucair-Jaafar et al., 2014). While chronic nortriptyline antiallodynic action was lost in DOP-deficient mice, this action remained fully present in MOP and in KOP deficient mice (Bohren et al., 2010; Megat et al., 2015). Even though an intact opioid system, at least for DOP receptors, appears to be necessary for the antiallodynic effect of antidepressants, the link between monoaminergic and opioid systems remains unclear.

Antidepressant treatment may increase the production of opioid peptides. For example, it has been shown in non-painful animals that a chronic treatment with amitriptyline increases enkephalin levels in the spinal cord and in some supraspinal structures (Hamon et al., 1987). A more recent study using microarray analysis also showed that this TCA increases proenkephalin and prodynorphin mRNA levels in the nucleus accumbens in naive mice (Bohm et al., 2006). Moreover, noradrenaline, through a β adrenoceptor-mediated mechanism, may also favor β-endorphin production by non-neuronal immune cells (Binder et al., 2004), which is abolished after chemical sympathectomy. Antidepressant treatment may also indirectly affect opioid receptors, indeed repeated administration of amitriptyline increases the densities of MOP and DOP binding sites in the spinal cord (Hamon et al., 1987). However, the impact of chronic antidepressant treatment on the opioid system in neuropathic pain conditions is still to be addressed.

The respective location of adrenergic and opioid receptors is a critical point. Indeed, if both receptors were to be expressed by the same cells, direct interactions might be

possible. In cell culture, coexpression of DOP or KOP receptors and of β2 adrenoceptors can lead to heteromerization of these receptors at the membrane (Jordan et al., 2001). Similarly, co-expressed MOP receptors and α2A adrenoceptors can physically interact, which impacts on their intracellular signaling (Jordan et al., 2003). Using FRET approaches, a functional cross-talk between these two receptors, with inhibition of one receptor by the other, has been proposed (Vilardaga et al., 2008). On the other hand, if adrenergic and opioid receptors are on different cells, a cascade mechanism implying opioid peptide synthesis and/or opioid receptor regulation (as described above), would be more likely. It is thus important to identify to which extent these receptors are or are not co-expressed in the nociceptive system.

Neuroimmune component

Accumulating evidence supports the involvement of immune and neuroimmune actors in neuropathic pain pathophysiology (Austin and Moalem-Taylor, 2010). This implication is also referred to as neurogenic inflammation, which concerns for instance the recruitment of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL) -6 and IL-1β (Sorkin et al., 1997; Zhang et al., 2002; Leung and Cahill, 2010; Mika et al., 2015), which might be impacted by antidepressant treatment. Indeed, spinal nerve ligation increases brain levels of TNF-α and IL-1β levels, which are reduced after repeated mirtazapine administration (Zhu et al., 2008). In fact, this antidepressant treatment favors the production of the anti-inflammatory cytokine IL-10, while blunting the hyperactivity of NF-κB (Zhu et al., 2008), a transcription factor known to favor TNF-α production. After sciatic nerve constriction, amitriptyline has more precisely been shown to decrease TNF-α immunoreactivity in both the hippocampus and injured peripheral nerves (Sud et al., 2008), which has been suggested to be related to a change in α2 adrenoceptors coupling to inflammatory processes. While chronic treatment with nortriptyline or venlafaxine also suppress neuropathic pain-induced overexpression of TNF-α in dorsal root ganglia satellite cells, the use of knock-out mice demonstrated that this mechanism is in fact β2 adrenoceptor-mediated (Bohren et al., 2013). Overall, these studies converge to support the idea that antidepressant drugs can attenuate proinflammatory cytokine production in neuropathic pain, even though the exact mechanism is still to be detailed.

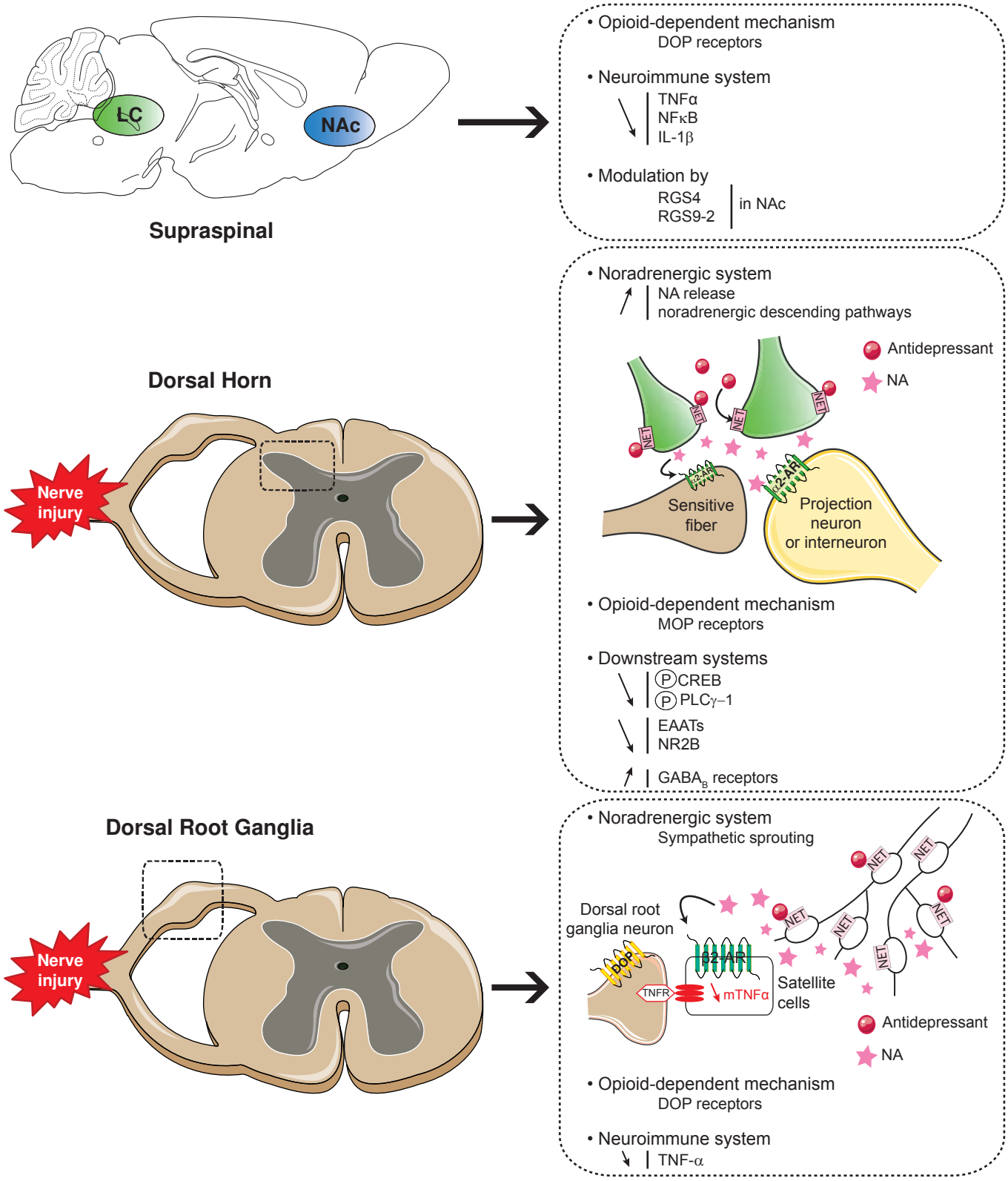

Fig. 1. Antidepressant drugs' action on neuropathic pain: insights from animal research.
The noradrenergic system is critical to the action of antidepressant drugs on neuropathic pain. There is research evidence for both a potential centrally mediated action implying descending controls of nociception, and a potential peripheral mechanism via an activation of β2-adrenoceptors in dorsal root ganglia. Abbreviations: α2-AR, α2 adrenoceptor; β2-AR, β2 adrenoceptor; CREB, cAMP response element-binding protein; DOP, delta opioid receptor; EAAT, excitatory amino acid transporters; GABA, gamma-aminobutyric acid; IL, interleukin; LC, locus coeruleus; MOP, mu opioid receptor; NA, noradrenaline; NAc, nucleus accumbens; NET, noradrenaline transporter; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NR2B, N-methyl D-aspartate receptor subtype 2B; PLCγ, phosphoinositide phospholipase γ; RGS, regulators of G protein signaling; TNF-α, tumor necrosis factor α; TNFR, TNF-α receptor.

Excitatory and inhibitory transmission

Central sensitization, which represents an enhancement in the function of neurons and circuits in nociceptive pathways, is an important component of neuropathic pain (Baron, 2006; Latremoliere and Woolf, 2009). Antidepressants might impact on such mechanism either directly or indirectly. Directly, *in vitro* studies have evidenced that TCAs have some affinity for NMDA receptors (Reynolds and Miller, 1988), amitriptyline appears for example to act as a non-competitive antagonist of NMDA receptor-mediated events (Kiefer et al., 1999). However, it is not clear whether these properties are relevant at clinical doses. Indirectly, either amitriptyline or mirtazapine have been shown to attenuate oxaliplatin-induced up-regulation of NR2B in the spinal cord (Sada et al., 2012; Liu et al., 2013); and amitriptyline may favor the spinal expression of excitatory amino acid transporters following spared nerve injury (Mao and Yang, 2010). It has also been proposed that a deficit in spinal inhibitory transmission may participate to neuropathic pain (Ueda, 2006; Latremoliere and Woolf, 2009; Prescott, 2015). At early stage following sciatic nerve ligation, amitriptyline treatment blocks the changes in dorsal horn $GABA_B$ receptor function (McCarson et al., 2005). However, further studies will be necessary to assess whether an impact of antidepressants on excitatory and/or inhibitory transmission might be relevant to their therapeutic action in neuropathic pain.

Other

While antidepressants promote the activity of the transcription factor cyclic adenosine monophosphate-responsive element-binding protein (CREB) in limbic structures (Thome et al., 2000; Nestler et al., 2002), long-term imipramine treatment has a different impact on the spinal cord of mice with partial sciatic nerve ligation (Kusuda et al., 2013). This TCA markedly reduces CREB phosphorylation and PLCγ-1 phosphorylation, two upregulated parameters following nerve injury, in the dorsal horn ipsilateral to the ligation (Kusuda et al., 2013). At supraspinal level, continuous delivery of milnacripran in rats with sciatic nerve constriction suppresses nociceptive-induced activation (Fos) of the anterior cingulate cortex (Takeda et al., 2009), a cortical region that is critical to the aversiveness of spontaneous pain (Johansen et al., 2001; Qu et al., 2011; Barthas et al., 2015) and to the anxiodepressive consequences of neuropathic pain (Barthas et al., 2015). Beside behavioral effects, these findings illustrate that antidepressants can also reverse some molecular aspects of neuropathic pain.

Table 3. Proposed mechanisms of action of the analgesic effect of gabapentinoids. *Abbreviations:* AR, adrenoceptor; BDNF, brain-derived neurotrophic factor; CCI, chronic constriction injury; d, days; DA, dopamine; DRG, dorsal root ganglia; EAAT, excitatory amino acid transporters; GABA, gamma-aminobutyric acid; i.c.v, intracerebroventricular; i.p, intraperitoneal; i.t., intrathecal; i.v. intravenous; LC, locus coeruleus; MHPG, 3-methoxy-4-hydroxyphenylglycol; NA, noradrenaline; NAc, nucleus accumbens; NGF, nerve growth factor; OXP, oxaliplatin; PAG, periaqueductal gray; pCREB, phosphorylated cAMP response element-binding protein; PCX, paclitaxel; PKA, protein kinase A; p.o, per os; PSNL, partial sciatic nerve ligation; s.c, subcutaneous; SNI, spared nerve injury; SNL, spinal nerve ligation; SNT, spinal nerve transection; STZ, streptozotocin; SuNI, sural nerve injury; TRT, treatment; VDCC, voltage-dependent calcium channel; VIN, vincristine; VZV, varicella zoster virus; w, weeks; WDR, wide dynamic range.

GABAPENTIN

Dose (mg/kg)	TRT	Species	Pain models	Pain parameters	Comments	References
10 to 1000 µg (i.t.)	-	Rat	SNL	Mechanical allodynia ↓	Dose response effect, does not involve $GABA_A$ or $_B$ receptors	Hwang and Yaksh (1997)
90 (i.v.)	-	Rat	PSNL	Mechanical allodynia ↓	Inhibition of peripheral ectopic afferent discharge activity in injured nerve site	Pan et al. (1999)
30, 100 (i.c.v., i.t., i.p.)	-	Mice	PSNL	Mechanical allodynia ↓ Thermal hyperalgesia ↓	Involvement of descending noradrenergic system and α2-ARs	Tanabe et al. (2005)
30 to 300 (i.p.) 0.42 to 4.2 (i.t.)	-	Rat	CCI	Mechanical allodynia ↓ Cold hyperalgesia ↓	Reduced formalin-induced release of EAATs in the spinal cord dorsal horn	Coderre et al. (2005)
10, 30, 100 (s.c.)	-	Rat	SNL	Mechanical allodynia ↓ Cold hyperalgesia ↓	Activation of spinal $5HT_3$ receptors	Suzuki et al. (2005)
50 (i.p.)	6d	Mice	PSNL	Mechanical allodynia ↓ Thermal hyperalgesia ↓ Thermal allodynia ↓	CB1 cannabinoid receptors are not involved in the effects of gabapentin	Castane et al. (2006)
3, 10 and 30 (i.p.)	-	Mice	PCX	Mechanical allodynia ↓ Thermal hyperalgesia ↓	Dose dependent Block A-fiber hypersensitization	Matsumoto et al. (2006)
50 (i.p.), 10 to 30 (i.t.)	-	Mice	SNL	Tactile allodynia ↓ *Dose dependent*	Inhibition of high threshold VDCC current in DRG neurons mediated by increased α2δ1 subunit expression	Li et al. (2006)
100, 300 (s.c.)	-	Mice	CCI	Mechanical allodynia ↓	Action mediated through α2δ1 subunit of VDCC	Field et al. (2006)
100 (i.p.)	4d	Mice	PCX	Mechanical allodynia ↓	Decrease the increased α2δ1 subunit expression in spinal dorsal horn induced by paclitaxel	Xiao et al. (2007)
100, 300 µg (i.c.v.)	-	Mice	PSNL	Mechanical allodynia ↓	Increase spinal MHPG and MHPG/NA concentrations Analgesic effects mediated supraspinally	Takeuchi et al. (2007a)
30 to 300 µg (i.c.v)	-	Mice	PSNL	Mechanical allodynia ↓ Thermal hyperalgesia ↓	Increase noradrenergic turnover in the spinal cord Involvement of spinal α2-ARs but not α1-ARs Decreases GABA activity in the LC	Tanabe et al. (2008)
50 (i.v.) 0.1 to 3 µg (LC)	-	Rat	SNL	Mechanical allodynia ↓	Induction of pCREB activation in LC neurons via AMPA receptors; NA release in the spinal dorsal horn; Involvement of spinal α2-ARs	Hayashida et al. (2008)
50 (p.o.)	5d	Rat	STZ	Mechanical allodynia ↓	Attenuation of microglial activation Unaffected astrocytic activation	Wodarski et al. (2009)
3 to 30 (s.c.)	-	Mice	SuNI	Mechanical hyeralgesia↓ Cold hyperalgesia ↓	Inhibition of spontaneous activity of WDR neurons in the lumbar dorsal horn	Omori et al. (2009)
100 µg (i.c.v.)	-	Mice	PSNL	Mechanical hyeralgesia↓ Thermal hyperalgesia ↓	Analgesic effects is mediated by supraspinal PKA and GABAergic inhibition of LC neurons	Takasu et al. (2009)
50 (i.p.)	3d	Rat	STZ	Mechanical hyeralgesia↓	Decrease Fos expression in the PAG	Morgado et al. (2010)
10 to 100 (i.t), 50 (s.c.)	-, 3, 4 or 7d	Mice	SNL	Mechanical allodynia ↓	Decrease the increased α2δ1 subunit expression in spinal dorsal horn induced by SNL	Morimoto et al. (2012)
30 µg (i.t.)	7d	Rat	SNL	Mechanical allodynia ↓	Reduce the expression of pro-inflammatory (TNF-α, IL-1β, IL-6) and increase the expression of IL-10 in the dorsal horn	Lee et al. (2013)
30 to 120 (p.o.)	5d	Rat	CCI	Thermal hyperalgesia ↓	Enhance TNF-α and IL-1β levels and decrease IL-10 levels in nerve	Camara et al. (2013)
300 (p.o.)	-	Rat	SNL	Mechanical allodynia ↓	Increase DA in the NAc	Xie et al. (2014)
60 (i.p.)	14d	Rat	OXP	Mechanical allodynia ↓	Prevent increased BDNF expression in the dorsal horn; Reduce glutamatergic transmission	Ruyang et al. (2015)

To date, a majority of the published data concerning chronic treatment with antidepressants in a neuropathic pain context focused on spinal mechanisms, with fewer information on peripheral (Zhu et al., 2008; Bohren et al., 2013) or supraspinal (Marchand et al., 2003b; Sud et al., 2008; Zhu et al., 2008; Stratinaki et al., 2013; Mitsi et al., 2015) aspects. Since we chose to focus the present review on data obtained from sustained or chronic treatments with antidepressants, a large part of the animal-based literature concerning acute antidepressants' effects was put aside (for review, see Mico et al., 2006). While focusing on chronic treatments seems to be consistent with clinical data, it has not yet been demonstrated in animal models whether the mechanisms of action underlying acute and chronic administration of antidepressants would be similar or different.

GABAPENTINOID DRUG MECHANISMS IN NEUROPATHIC PAIN

Gabapentinoid anticonvulsants (Field et al., 2007), which include gabapentin and pregabalin, have proved to be clinically effective in a number of neuropathic pain conditions (Finnerup et al., 2015). Similarly, gabapentinoids are effective in many murine models of neuropathic pain. In addition to the models already described above for antidepressants' results, chronic treatment studies have also been conducted in chemotherapy-induced neuropathic pain [vincristine (Higuera and Luo, 2004) and paclitaxel (Polomano et al., 2001)], and in a model of sural nerve injury (Omori et al., 2009). The possible mechanisms involved in the therapeutic action of gabapentinoids have been actively studied (Cheng and Chiou, 2006), these studies being mainly conducted with a single administration of gabapentinoids (**Table 3**). In the pain field, both clinical and preclinical studies show for example an acute effect of gabapentin or pregabalin administration at mild to high dose (Wallin et al., 2002; Tanabe et al., 2005; Field et al., 2006; Takeuchi et al., 2007b; Benbouzid et al., 2008a; Xie et al., 2014; Wang et al., 2015), although the long-term effect at mild doses may more likely be representative of the widespread clinical use of gabapentinoids in neuropathic pain. However, only a few studies have investigated the mechanisms of long-term treatment with gabapentinoids (**Table 3**).

Although structurally related to GABA, gabapentinoids do not primarily target GABA receptors. The identification of a specific binding site on the $\alpha_2\delta$ subunit of voltage-

PREGABALIN						
30 and 100 (s.c.)	-	Mice	CCI	Mechanical allodynia ↓	Action mediated through α2δ1 subunit of VDCC	Field et al. (2006)
10, 30 (i.p.) 10, 30µg (i.c.v, i.t.)	-	Mice	PSNL	Mechanical allodynia ↓ Thermal hyperalgesia ↓ ↔ *just for 10 i.p.*	Increase spinal MHPG & MHPG/NA concentrations. Analgesic effects mediated supraspinally and spinally. Involvement of spinal α2-ARs but not α1-ARs	Takeuchi et al. (2007b)
10 to 100 µg (i.c.v)	-	Mice	PSNL	Mechanical allodynia ↓ Thermal hyperalgesia ↓	Increase the noradrenergic turnover in the spinal cord Involvement of spinal α2-ARs but not α1-ARs	Tanabe et al. (2008)
30 (s.c.)	8d	Rat	SNL	Mechanical allodynia ↓ Cold hyperalgesia ↓	Reduce the elevation of α2δ1 subunit in the spinal cord and ascending axon tracts. Inhibit trafficking of α2δ1 subunits from the DRG cell bodies to spinal presynaptic terminals	Bauer et al. (2009)
30 (p.o.)	4d	Mice	CCI	Mechanical allodynia ↓	Suppress immune-related impact of CCI	Jang et al. (2012)
300, 900 µg (i.t.)	7d or 28d	Rat	SNI	Mechanical allodynia ↓ Mechanical hyperalgesia↓ Cold hyperalgesia ↓	Do not inhibit trafficking of α2δ1 subunit nor the upregulation of spinal levels of this subunit	Yang et al. (2014)
100 µg (i.t.)	-	Rat	SNI	Mechanical allodynia ↓	Inhibition of spontaneous activity of WDR neurons in the lumbar dorsal horn	Ding et al. (2014)
12.5, 25 (p.o.)	-	Mice	PSNL	Mechanical allodynia ↓ Thermal hyperalgesia ↓	Reverse the increase of c-Fos expression in neurons of the anterior cingulate cortex	Wang et al. (2015)

dependent calcium channels (VDCCs) (Li et al., 2011; Zamponi et al., 2015), suggested that gabapentinoids' antinociceptive action was mediated through an attenuation of Ca^{2+} channels influx into cells (Gee et al., 1996; Field et al., 2006; Matsuzawa et al., 2014).

Voltage-dependent Ca^{2+} channels in neuropathic pain

In various animal models of neuropathic pain, an increased expression of $\alpha_2\delta$ calcium channel subunit mRNA and/or protein is observed in the dorsal root ganglia and/or in the dorsal horn of the spinal cord (Luo et al., 2001; Luo et al., 2002; Narita et al., 2007; Xiao et al., 2007). Such modification is however not generalized to all painful neuropathic conditions. For example, there is no change in $\alpha_2\delta$ subunit expression in vincristine-induced painful neuropathy (Luo et al., 2002). The increase in $\alpha_2\delta$ subunit in the spinal cord has been proposed to result from an elevation within the presynaptic terminals of primary afferent neurons, rather than from overexpression in intrinsic dorsal horn neurons (Narita et al., 2007; Bauer et al., 2009). In mice engineered to overexpress $\alpha_2\delta$ subunit, tactile allodynia is present in the absence of nerve damage (Li et al., 2006). On the contrary, $\alpha_2\delta$ knock-out mice show reduced behavioral sensitivity to nociceptive mechanical and cold stimuli (Patel et al., 2013). While there is a substantial body of evidence showing that levels of $\alpha_2\delta$ subunits affect nociceptive sensitivity as well as the development of neuropathic pain, the therapeutic action of gabapentinoids does not require increased $\alpha_2\delta$ subunits levels to be effective against pain.

Repeated administration of gabapentin or pregabalin normalizes the increased spinal $\alpha_2\delta$ levels produced by damage to primary afferent sensory neurons (Xiao et al., 2007; Bauer et al., 2009; Morimoto et al., 2012). This effect is even present while chronic pregabalin does not impact on $\alpha_2\delta$ mRNA and protein levels in the dorsal root ganglia, which indicates that it is not the expression of the $\alpha_2\delta$ subunit but rather its trafficking from the dorsal root ganglia cell bodies to spinal presynaptic terminals that is affected by gabapentinoids (Bauer et al., 2009). Indeed, sustained gabapentin treatment markedly reduces cell surface localization of $\alpha_2\delta$ subunit, both in cellular expression systems and in dorsal root ganglia neurons (Hendrich et al., 2008). Although these data point towards a therapeutic mechanism in which sustained gabapentinoids would inhibit trafficking of $\alpha_2\delta$ subunits, it should be noted that a recent study reported a pain-relieving action of chronic intrathecal infusion of pregabalin in the absence of detectable change in spinal levels of the $\alpha_2\delta$ subunit (Yang et al., 2014).

The $\alpha_2\delta$ subunit is not only expressed at the peripheral and spinal levels where its expression sometimes undergoes changes with the induction of neuropathic pain, but it is also expressed supraspinally. For example, within areas of the brain associated with nociceptive processing, a moderate to strong expression of the $\alpha_2\delta$ subunit has been reported in the dorsal raphe, periaqueductal gray, locus coeruleus and amygdala (Cole et al., 2005; Taylor and Garrido, 2008; Mico and Prieto, 2012). Gabapentinoids may therefore have direct action on various brain regions, which is supported by human imaging data (Aupperle et al., 2011; Harris et al., 2013), even though these supraspinal effects are more rarely studied in animal models. A study using 18 F-fluorodeoxyglucose-positron emission tomography scanning in rats with spared nerve injury showed that gabapentin analgesic effect may be mediated by reversing central hypersensitivity and suppressing medial prefrontal cortical activity, a crucial part of the cortical representation of pain in the brain (Lin et al., 2014). Other information on this supraspinal action will be developed in the sections that follow.

Sensitization

After peripheral nerve injury, damaged and non-damaged A-fibers can generate spontaneous action potentials and central sensitization develops in the dorsal horn (Latremoliere and Woolf, 2009). Gabapentinoids will impact on both aspects. Acutely, gabapentin can reduce the ectopic discharges and A-fiber hypersensitization induced by partial ligation of the sciatic nerve (Pan et al., 1999) or by Paclitaxel administration (Matsumoto et al., 2006). Studies conducted *ex vivo* show that sustained exposure to gabapentinoids reduces calcium channel currents in dorsal root ganglia neurons (Hendrich et al., 2008), but targets preferential neuronal subpopulations (Biggs et al., 2014). This leads to stronger impact on spontaneous excitatory postsynaptic currents in the putative excitatory neurons rather than putative inhibitory neurons of the substantia gelatinosa (Biggs et al., 2014). *In vivo*, gabapentinoids will inhibit the hyperactivity of dorsal horn wide-dynamic range neurons in different models of neuropathic pain (Omori et al., 2009; Ding et al., 2014), and reduce wind-up in rats with mononeuropathy (Curros-Criado and Herrero, 2007). This action may in part result from decreased excitatory aminoacid release, as suggested by the observation that gabapentin inhibits formalin-induced increase in spinal aspartate and

glutamate release in a chronic constriction injury model of neuropathic pain (Coderre et al., 2005).

The impact of gabapentinoids is also found at supraspinal levels. Following spinal nerve ligation in rodents, acute systemic gabapentinoid decreases the elevated spontaneous and stimulus-evoked activities displayed by neurons of the central nucleus of the amygdala (Goncalves and Dickenson, 2012). Gabapentin also down-regulates the hyperactivity state of supraspinal sensorimotor systems, in particularly within networks implying limbic structures from the cortex and the hippocampal formation (Hooker et al., 2014).

Moreover, gabapentinoid treatment suppresses nociceptive-induced activation (Fos) of the periaqueductal grey matter and of the anterior cingulate cortex (Morgado et al., 2010; Wang et al., 2015). In line with behavioral effects, these findings illustrate that gabapentinoids can also reverse neuronal hyperactivity induced by neuropathic pain.

Aminergic systems

While an action on the trafficking of $\alpha_2\delta$ subunits in primary afferents appears particularly important for the action of gabapentinoids after sustained treatment, a supraspinal action on descending controls of pain has been suggested for their acute action at high dose. Indeed, gabapentinoids could recruit the descending noradrenergic pathway that originates from the locus coeruleus. For instance, gabapentin activates locus coeruleus neurons (Hayashida et al., 2008; Suto et al., 2014a), the intracerebroventricular delivery of high doses of gabapentinoids increases noradrenergic turnover in the spinal cord (Takeuchi et al., 2007a; Takeuchi et al., 2007b; Tanabe et al., 2008), and the intravenous administration of a high dose of gabapentin increases spinal cord extracellular levels of noradrenaline (Hayashida et al., 2008). In human, 1.2 g of gabapentin increases noradrenaline levels in the cerebrospinal fluid (Hayashida et al., 2007). The functional importance of this recruitment is further evidenced by the fact that a noradrenergic lesion or an α2, but not an α1, adrenoceptor antagonist almost suppresses the acute action of gabapentinoids in the partial sciatic nerve ligation model of neuropathic pain (Tanabe et al., 2005; Takeuchi et al., 2007b; Tanabe et al., 2008). The action of gabapentinoids on the descending pathways would result from PKA-dependent decreased GABA activity (Takasu et al., 2008; Tanabe et al., 2008; Takasu et al., 2009; Yoshizumi et al., 2012), and from increased glutamatergic levels (Hayashida et al., 2008; Suto et al., 2014b) in the locus coeruleus. Interestingly,

gabapentinoid recruitment of locus coeruleus cells in a neuropathic pain condition might selectively concern the descending pathway, but not ascending projections to the prefrontal cortex (Suto et al., 2014a). This mechanism recruiting descending pathways has been observed at acute high doses of gabapentinoids. However, it has not yet been assessed whether it also participates to the therapeutic effect present at milder doses with long term treatment.

Beside the implication of the noradrenergic system, it has also been suggested that descending serotonergic transmission could be important for acute gabapentinoid action in a neuropathic pain context (Suzuki et al., 2005; Bee and Dickenson, 2008), and it has been shown that pain relieving action of a high dose of gabapentin in rats with spinal nerve ligation is accompanied by increased extracellular levels of dopamine in the nucleus accumbens shell (Xie et al., 2014).

Neuroimmune component

Some data suggest that gabapentinoids can affect glial cells and expression of proinflammatory cytokines, which may be a secondary downstream consequence of gabapentinoid treatment. In the dorsal horn of the spinal cord, increased expression of a marker of microglia, CD11b, following spinal nerve ligation is not attenuated by sustained administration of gabapentin (Morimoto et al., 2012). However, gabapentin reverses the increased microglial activation, as shown by studying Iba-1 expression in the spinal cord of rats with streptozotocin-induced neuropathic pain (Wodarski et al., 2009). It also reverses oxalipatin-induced brain-derived neurotrophic factor expression in the spinal cord (Ruyang et al., 2015). Gabapentinoid treatment can also impact on cytokine overexpression. Thus, pregabalin suppresses the dorsal root ganglia overproduction of TNF-α induced by sciatic nerve constriction (Kremer et al., 2016). In the spinal cord, increased levels of TNF-α, IL-6 and IL-1β following spinal nerve ligation are also reversed by repeated intrathecal gabapentin administrations, an effect that may be due, at least in part, to the upregulation of the anti-inflammatory cytokine IL-10 (Lee et al., 2013). However, it is to be noted that a study found that gabapentin enhanced TNF-α and IL-1β levels and decreased IL-10 levels in injured sciatic nerve when given during the first days post-surgery (Camara et al., 2013). This is surprising as long-term pregabalin treatment also suppresses other immune-related

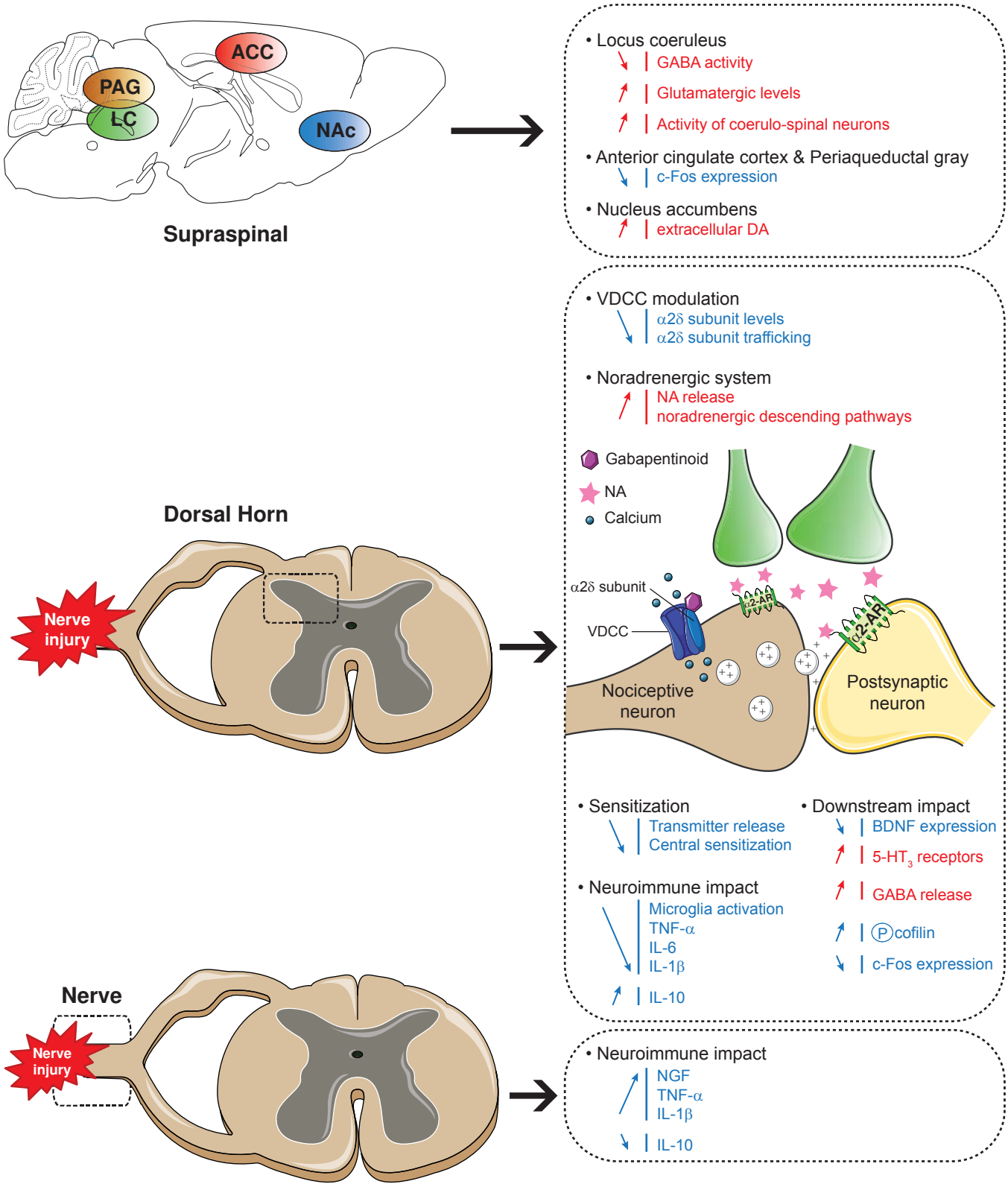

Fig. 2. Gabapentinoids' action on neuropathic pain: insights from animal research.
In red are shown the events after acute administration of gabapentin or pregabalin; and in blue those associated with prolonged treatment. The primary target of gabapentinoids is the voltage-dependent calcium channels α2δ-1 subunit. Gabapentinoids' binding to these subunits reduces excitatory transmitter release and spinal sensitization. Secondary mechanisms, such as the activation of the descending noradrenergic pain inhibitory system or the decrease of microglial activation and/or proinflammatory cytokines, may also be involved in the relief of neuropathic pain by these drugs. Abbreviations: α2-AR, α2 adrenoceptor; ACC, anterior cingulate cortex; BDNF, brain-derived neurotrophic factor; DA, dopamine; DRG, dorsal root ganglia; GABA, gamma-aminobutyric acid; IL, interleukin; LC, locus coeruleus; NA, noradrenaline; NAc, nucleus accumbens; NGF, nerve growth factor; PAG, periaqueductal gray; TNF-α, tumor necrosis factor α; VDCC, voltage-dependent calcium channels.

impact of peripheral neuropathy, such as the activated natural killer cell activity and lymphocyte proliferation accompanying chronic constriction injury in mice (Jang et al., 2012).

GABAergic system

Gabapentin and pregabalin do not bind $GABA_A$ or $GABA_B$ receptors, and do not interact with GABA uptake transporters (Lanneau et al., 2001; Li et al., 2011; Mico and Prieto, 2012), and the acute antiallodynic action of intrathecal gabapentin remains effective in the presence of intrathecal $GABA_A$ or $GABA_B$ receptor antagonists (Hwang and Yaksh, 1997). However, gabapentinoid treatment has an indirect inhibitory effect on GABAergic transmission in the locus coeruleus (see above **Aminergic systems** section); while systemic administration of gabapentin increases spinal GABA release in rats with spinal nerve ligation (Yoshizumi et al., 2012).

Opioid and cannabinoid systems

Two recent studies suggested that gabapentinoids might recruit the endogenous opioid system (Kaygisiz et al., 2015; Miranda et al., 2015). Indeed, naloxone reversed the acute antinociceptive activity of a high dose of pregabalin in naive mice (Kaygisiz et al., 2015); and naltrexone affected acute gabapentin action in a model of orofacial inflammatory pain (Miranda et al., 2015). However, these data differ from all previous studies on gabapentinoids, which reported opioid antagonists to be ineffective in blocking gabapentinoid-induced analgesia in different pain models (Field et al., 1997; Eutamene et al., 2000; Benbouzid et al., 2008a). In a context of neuropathic pain, the acute and the chronic antiallodynic effect of gabapentinoid is not inhibited by an opioid antagonist, and remains fully present in MOP-deficient, in DOP-deficient and in KOP-deficient mice (Benbouzid et al., 2008a; Kremer et al., 2016). This demonstrates that the opioid system is not necessary to gabapentinoid antiallodynic action in neuropathic pain. The opioid and cannabinoid systems are interdependent. Similarly to opioid receptors that are not required for pregabalin action, sustained gabapentin remains effective in CB1 cannabinoid receptor deficient mice (Castane et al., 2006).

Table 4. Compared mechanisms for antidepressants and gabapentinoids. *Abbreviations:* AR, adrenoceptor; DH, dorsal horn; DOP, delta opioid; DRG, dorsal root ganglia; EAAT, excitatory amino acid transporters; GABA, gamma-aminobutyric acid; IL, interleukin; MOP, mu opioid; n.d., not described; NA, noradrenaline; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; TNF, tumor necrosis factor.

	Antidepressants	Long-term gabapentinoids	Acute gabapentinoids
Monoaminergic component			
descending NA pathways	yes	n.d.	yes
peripheral NA	yes		n.d.
α_2-AR	yes		yes
β_2-AR	yes		n.d.
Opioidergic component			
DOP and/or MOP	yes	no	no
Neuroimmune component			
TNFα	yes (*supraspinal/DH/DRG*)	yes (*DH/nerve/DRG*)	n.d.
NFκB	yes (*supraspinal*)	n.d.	
IL-1β	yes (*supraspinal*)	yes (*DH*)	
IL-6	n.d.	yes (*DH*)	
IL-10	n.d.	yes (*DH/nerve*)	
microglia activation	n.d.	yes (*DH*)	
Excitatory and inhibitory transmission			
EAATs	yes (*DH*)	yes (*DH*)	yes (*supraspinal*)
central sensitization	yes (*DH*)	yes (*DH*)	n.d.
GABAergic system			
$GABA_B$ receptors	yes (*DH*)	n.d.	n.d.
GABA release	n.d.		yes (*supraspinal/DH*)
α2δ subunit modulation			
levels	no	yes	n.d.
trafficking	no	yes	

CONCLUSION

Even though their efficacy is in part limited, antidepressants and gabapentinoids have become common drugs for the treatment of neuropathic pain and remain the best pharmacological treatments to date. Preclinical research allowed progress in understanding the bases of their therapeutic action, but the detailed mechanism(s) of pain relief is(are) not yet fully described. While the primary targets of antidepressants and gabapentinoids profoundly differ, it is interesting to note that some aspects of their mechanisms might indirectly converge (**Table 4**). Research in neuropathic pain treatments evolved substantially in recent years. To improve efficacy, consistent clinical criteria based on signs and symptoms, etiology and severity of neuropathic pain, might be required for selecting the most appropriate treatment. Such strategy might however require a mechanism-based classification of neuropathic pain and appropriate markers, which still doesn't exist.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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TRAVAUX PERSONNELS

I. The antiallodynic action of pregabalin in neuropathic pain is independent from the opioid system.

Kremer M, Nexon L, Wurtz X, Ceredig RA, Daniel D, Hawkes R, Yalcin I, Salvat E, Barrot M. ***Mol Pain,*** 2016, 12:1744806916636387.

Les anticonvulsivants de la famille des gabapentinoïdes, dont les représentants sont la gabapentine et la prégabaline, sont des molécules dont l'efficacité est cliniquement prouvée pour le soulagement des douleurs neuropathiques de différentes étiologies (Finnerup et al., 2015). Ces molécules agissent sur la sous-unité α2δ des canaux calcium dépendants du voltage (Gee et al., 1996; Field et al., 2006), sous-unité largement exprimée au niveau des terminaisons pré-synaptiques de nombreux neurones, principalement excitateurs (Dolphin, 2012; Patel and Dickenson, 2016). Ainsi, il a été montré que les gabapentinoïdes inhibent les courants calcium, conduisant à une diminution de la transmission excitatrice (Mico and Prieto, 2012). Si ces dernières années de nombreuses avancées ont été faites dans la compréhension du mécanisme d'action des gabapentinoïdes sur la douleur neuropathique, certains aspects de cette action demandent encore à être clarifiés.

Une action synergique entre gabapentinoïdes et opioïdes contre la douleur neuropathique est régulièrement proposée en clinique comme en préclinique (De la O. Arciniega et al., 2009; Chaparro et al., 2012; Bao et al., 2014). Ainsi, il a été montré que la prégabaline augmente l'efficacité de la morphine et permet d'atténuer ses effets indésirables dose-dépendants chez des patients souffrant de douleurs neuropathiques post-chimiothérapie (Dou et al., 2014). Cette combinaison thérapeutique semble avoir un intérêt réel, mais deux questions au moins se posent. Cette synergie résulte-t-elle d'une convergence de mécanismes pouvant induire des effets additifs, de potentialisation ou même d'inhibition ? Ces molécules agissent-elles indépendamment sur les voies nociceptives, via des cibles différentes, conduisant à une complémentarité d'effets ? Pour le moment les données expérimentales ne permettent pas de répondre. L'aspect exploré à travers cet article concerne l'implication des récepteurs des opioïdes dans l'action antiallodynique de la prégabaline. En effet, de rares études ont suggéré un recrutement potentiel du système opioïdergique lors d'une administration aigüe à forte dose de gabapentinoïdes dans le soulagement d'une douleur induite par l'administration d'un agent

chimique (Miranda et al., 2015) ou par un stimulus nociceptif (Kaygisiz et al., 2015) chez des souris naïves. Dans ces deux études, l'utilisation de naloxone ou de naltrexone, des antagonistes non sélectifs des récepteurs des opioïdes, réverse les effets antinociceptifs de la prégabaline ou de la gabapentine, suggérant un rôle potentiel de ces récepteurs dans l'action aigüe de ces molécules. D'autres travaux n'ont par contre montré aucun effet de la naloxone sur le soulagement de la douleur par les gabapentinoïdes, et ce aussi bien dans un modèle de compression du nerf sciatique (Benbouzid et al., 2008a), que dans un modèle de douleur viscérale (Eutamene et al., 2000), ou encore dans un modèle de douleur aigüe suite à un l'application d'un stimulus nociceptif (Field et al., 1997).

A côté de cette action opioïdergique, nous avons aussi exploré l'incidence de la prégabaline sur un élément de la neuroinflammation, le TNFα. Comme pour les antidépresseurs, un impact potentiel des gabapentinoïdes sur l'expression de cytokines pro- ou anti-inflammatoires a en effet été évoqué par divers auteurs (Bao et al., 2014; Dias et al., 2014). Ainsi, une étude récente montre que des injections intrathécales répétées de gabapentine normalisent les taux de TNFα, IL-6 et IL-1β dans la moelle épinière, taux augmentés suite à une ligature du nerf spinal chez l'animal (Lee et al., 2013). Pourtant ce mode d'administration intrathécale n'est pas utilisé pour les gabapentinoïdes en clinique, où ils sont administrés en systémique, par voie orale. Il est alors intéressant de se rapprocher de la clinique et d'évaluer le lien entre la prégabaline et le TNFα, dans un contexte de douleur neuropathique, mais cette fois-ci avec un traitement prolongé par voie orale.

Dans cet article publié dans *Molecular Pain*, nous avons déterminé si un traitement aigu et / ou chronique par la prégabaline nécessite le recrutement des récepteurs MOP, DOP ou KOP. Cela a été fait grâce à des approches comportementales, génétiques et pharmacologiques. En utilisant notre modèle dit du « cuff », nous montrons qu'une administration aigüe de prégabaline à forte dose permet un soulagement transitoire de l'allodynie mécanique, alors qu'un traitement prolongé, par voie orale, à plus faible dose, permet un soulagement au long terme. L'efficacité thérapeutique de la prégabaline est conservée chez des animaux transgéniques déficients pour le récepteur MOP, le récepteur DOP ou le récepteur KOP. De plus, ces actions antiallodyniques aigües ou chroniques ne sont pas affectées par une administration aigüe de naloxone, un antagoniste non sélectif des récepteurs des opioïdes. Enfin, un traitement prolongé par la prégabaline permet de

normaliser les taux de mTNFα, augmentés chez les animaux neuropathiques dans les ganglions rachidiens.

L'ensemble de ces résultats montre que le système opioïdergique n'est pas nécessaire à l'action antiallodynique des gabapentinoïdes, mais que ceux-ci agissent aussi sur la composante neuroimmunitaire périphérique de la douleur neuropathique, via une diminution des taux de mTNFα.

Dans le cadre de ce travail, j'ai personnellement effectué les expériences traitant de l'effet aigu de la prégabaline chez les souris C57BL/6J et chez les animaux déficients pour les récepteurs MOP, DOP et KOP (**Figures 4 et 5 de l'article**). J'ai également réalisé les expériences de biologie moléculaire afin d'évaluer l'effet d'un traitement prolongé par la prégabaline sur les taux TNFα dans les ganglions rachidiens (**Figure 6 de l'article**). De plus, j'ai directement encadré les étudiants ou techniciens qui, sous ma direction, ont effectué les expériences de dose-réponse (**Figure 1 de l'article**) ainsi que celles traitant de l'implication des récepteurs des opioïdes dans l'action prolongée de la prégabaline (**Figures 2 et 3 de l'article**).

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The antiallodynic action of pregabalin in neuropathic pain is independent from the opioid system

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Abstract

Background: Clinical management of neuropathic pain, which is pain arising as a consequence of a lesion or a disease affecting the somatosensory system, partly relies on the use of anticonvulsant drugs such as gabapentinoids. Therapeutic action of gabapentinoids such as gabapentin and pregabalin, which act by the inhibition of calcium currents through interaction with the α2δ-1 subunit of voltage-dependent calcium channels, is well documented. However, some aspects of the downstream mechanisms are still to be uncovered. Using behavioral, genetic, and pharmacological approaches, we tested whether opioid receptors are necessary for the antiallodynic action of acute and/or long-term pregabalin treatment in the specific context of neuropathic pain.

Results: Using the cuff model of neuropathic pain in mice, we show that acute pregabalin administration at high dose has a transitory antiallodynic action, while prolonged oral pregabalin treatment leads to sustained antiallodynic action, consistent with clinical observations. We show that pregabalin remains fully effective in μ-opioid receptor, in δ-opioid receptor and in κ-opioid receptor deficient mice, either female or male, and its antiallodynic action is not affected by acute naloxone. Our work also shows that long-term pregabalin treatment suppresses tumor necrosis factor-α overproduction induced by sciatic nerve constriction in the lumbar dorsal root ganglia.

Conclusions: We demonstrate that neither acute nor long-term antiallodynic effect of pregabalin in a context of neuropathic pain is mediated by the endogenous opioid system, which differs from opioid treatment of pain and antidepressant treatment of neuropathic pain. Our data are also supportive of an impact of gabapentinoid treatment on the neuroimmune aspect of neuropathic pain.

Keywords

pregabalin, neuropathic pain, mechanical allodynia, opioid system, tumor necrosis factor-α, μ-opioid, δ-opioid, κ-opioid

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Background

Neuropathic pain is defined as a direct consequence of a lesion or disease affecting the somatosensory system.[1] It can result from a wide range of conditions including diabetes, nerve root compression, herpes zoster infection, cancer, stroke, thus affecting millions of persons worldwide. This complex syndrome involves maladaptive changes in injured sensory neurons and along the entire nociceptive pathway within the central nervous system.[2] The recommended pharmacotherapy for neuropathic pain includes the use of anticonvulsant drugs, such as the gabapentinoids, pregabalin, and gabapentin.[3]

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Despite their structural similarity to the inhibitory transmitter γ-aminobutyric acid (GABA), neither gabapentin nor pregabalin binds to $GABA_A$ or $GABA_B$ receptors or interact with GABA uptake transporters.[4,5] Their therapeutic effect is mediated through binding to the α2δ-1 subunit of voltage-dependent calcium channels (VDCCs).[6,7] The interaction between gabapentinoids and the α2δ-1 subunit inhibits calcium currents, thus decreasing excitatory transmitter release.[5] This subunit also plays a role in trafficking VDCC complexes to cell surface[8] and in synaptogenesis, and these functions are blocked by gabapentin.[9]

The opioid system is involved in the action of different pain medications. This implication concerns on one hand the direct analgesic action of opioids targeting the μ-opioid (MOP) receptor[10] and on the other hand the indirect requirement of opioid receptors for the action of antidepressants against neuropathic pain.[11–13] During the past decade, it has been preclinically and clinically proposed that gabapentinoids and opioid drugs can have a synergistic action in neuropathic pain.[14–17] However, this does not mean that gabapentinoids require the endogenous opioid system. A potential role of the opioid system has been recently suggested in the central, acute, analgesic effect of a high dose of pregabalin in the tail flick test in naive mice,[18] and in the antinociceptive response induced by acute gabapentin in a model of acute inflammatory pain, the orofacial formalin test in mice.[19] On the contrary, previous pharmacological studies reported no effect of opioid antagonists on gabapentinoid action.[20–22] For example, naloxone do not block acute pregabalin action on abdominal constrictions in the lipopolysaccharide (LPS)-induced rectal hypersensitivity model of visceral pain;[21] and naloxone do not block acute gabapentin action in the formalin test, a model of inflammatory pain.[22] However, these studies did not really model the specific clinical use of gabapentinoids, i.e. in a neuropathic pain context, and did not either address the consequences of a long-term treatment.

Gabapentinoids have also been proposed to act on inflammatory mechanisms. Gabapentin may, for example, decrease the expression of pro-inflammatory cytokines;[16,23,24] this action has been associated with an upregulation of the anti-inflammatory cytokine interleukin (IL)-10.[24] Interestingly, experimental evidence supports a role of glial and/or immune cells in the pathophysiology of neuropathic pain, particularly through the recruitment of cytokines.[25] In sustained neuropathic pain, some pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) still display enhanced expression,[26–29] and blocking TNF-α has been preclinically postulated to relieve neuropathic pain symptoms.[26,30] It is, however, not known whether the expression of TNF-α is also targeted by pregabalin in a context of neuropathic pain.

In the present study, we used both genetic and pharmacological approaches to evaluate whether opioid receptors are critical for the antiallodynic action of acute and/or long-term pregabalin treatment. We demonstrate that neither the acute nor the long-term antiallodynic effect of pregabalin requires the endogenous opioid system. We also show that long-term pregabalin treatment inhibits the neuropathy-induced TNF-α overproduction in dorsal root ganglia (DRG).

Methods

Animals

Experiments were performed using male C57BL/6J mice (Charles River, L'Arbresle, France) with ages between 8 and 10 weeks at surgery time, or with mice lacking μ-opioid (MOP), δ-opioid (DOP), or κ-opioid (KOP) receptors and their littermate controls. The generation of mice lacking MOP, DOP, or KOP receptors has been previously described.[31–33] All mice were under a C57BL/6J background for over 10 generations. Heterozygote mice were bred in our animal facilities (breeders were kindly provided by Pr Kieffer and Pr Gavériaux-Ruff), genotyping of the litters was done, and the experiments were conducted on adult male and female wild type and knockout littermate mice weighing 20–30 g. We used the same number of males and females in each experimental group. As the wild type animals have the same background and the same behavior, they were pooled to form the control groups. Mice were group housed two to five per cage and kept under a 12 hr light/dark cycle with food and water *ad libitum*. A total of 104 C57BL/6J mice, 43 MOP-related, 43 DOP-related, and 43 KOP-related transgenic mice were used for the experiments. All animals received proper care in agreement with European guidelines (EU 2010/63). At the end of the experiments, mice were killed by cervical dislocation for immunoblot experiments, or by CO_2 inhalation (CO2 Euthanasia programmer 6.5 version, TEMSEGA, Pessac, France) followed by cervical dislocation for other experiments, according to the institutional ethical guidelines. The animal facilities Chronobiotron UMS3415 are registered for animal experimentation under the Animal House Agreement A67-2018-38. All protocols were approved by the "Comité d'Ethique en Matière d'Expérimentation Animale de Strasbourg" (CREMEAS, CEEA35).

Model of neuropathic pain

Neuropathic pain was induced by cuffing the main branch of the right sciatic nerve.[34,35] Surgeries were performed under ketamine (68 mg/kg)/xylazine (10 mg/kg)

intraperitoneal (i.p.) anesthesia (Centravet, Tadden, France). The common branch of the right sciatic nerve was exposed and a cuff of PE-20 polyethylene tubing (Harvard Apparatus, Les Ulis, France) of standardized length (2 mm) was unilaterally inserted around it (Cuff group). The shaved skin was closed using suture. Sham-operated mice underwent the same surgical procedure without implantation of the cuff (Sham group).

Measure of mechanical allodynia

Mechanical allodynia was tested using von Frey hairs, and results were expressed in grams. Tests were done during the morning, starting at least 2 hr after lights on. Mice were placed in clear Plexiglas boxes (7 cm × 9 cm × 7 cm) on an elevated mesh screen. Calibrated von Frey filaments (Bioseb, Vitrolles, France) were applied to the plantar surface of each hind-paw until they just bent, in a series of ascending forces up to the mechanical threshold. Filaments were tested five times per paw, and the paw withdrawal threshold (PWT) was defined as the lower of two consecutive filaments for which three or more withdrawals out of the five trials were observed.[35–37] The person who conducted the tests was blinded to the treatments.

Treatment procedures

The long-term treatment with pregabalin began two weeks after the surgical procedure (cuff implantation or sham surgery). Pregabalin (Lyrica®, Pfizer, Sandwich, UK), 300, 100, 50, or 5 μg/mL, was delivered per os through the drinking water with ad libitum access as sole source of fluid. This anticonvulsant drug was dissolved in water with 0.02% saccharin to increase palatability, and control mice were given a solution of 0.02% saccharin in water (vehicle solution). For acute administration, pregabalin was dissolved in 0.9% NaCl and administered intraperitoneally (30 mg/kg, 5 mL/kg). The injection of naloxone hydrochloride (Sigma–Aldrich, St. Quentin Fallavier, France), a competitive non selective MOP, DOP, and KOP receptors antagonist at high dose, was performed 25 days after surgery, i.e. after 11 days of pregabalin treatment; or 30 min after the acute administration of pregabalin. Naloxone hydrochloride was dissolved in 0.9% NaCl and administered subcutaneously (s.c., 1 mg/kg, 5 mL/kg). Long-term and acute treatment experiments were conducted on independent sets of mice.

Immunoblot analysis

In a separate experiment, DRG were collected from Sham-vehicle, Cuff-vehicle, and Cuff-pregabalin (300 μg/mL) group after two weeks of oral treatment. Mice were killed by cervical dislocation, the back was dissected, and a midline incision was done in the lumbar vertebrae to extract the L4, L5, and L6 DRG ipsilateral to the surgery. The three DRG were pooled per animal, quickly frozen, and stored at −80°C until protein extraction.

Total proteins were extracted in 150 μL lysis buffer (20 mM Tris pH 7.5; 150 mM NaCl; 10% glycerol; 1% NP-40; Protease Inhibitors Cocktail, Roche), quantitated with Bio-Rad Protein Assay Dye Reagent Concentrate and stored in Laemmli buffer (2% sodium dodecyl sulfate (SDS); 25% glycerol; 0.01% bromophenol blue; 0.125 M Tris pH 6.8); 10 μg of total protein from individual animals was resolved by 12% SDS-polyacrylamide gel electrophoresis under reducing conditions, and then transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon, transfer membranes, Millipore, IPVH00010). The blots were incubated for 1 h in blocking agent (ECL kit, Amersham Biosciences), overnight with the antibodies specific for either TNF-α (1:500, R&D Systems, AF-410-NA) or β-tubulin (1:50,000, Abcam, ab108342), followed by rabbit anti-goat horseradish peroxidase (HRP)-conjugated secondary antibodies (1:12,000, Abcam, ab97100) or goat anti-rabbit HRP-conjugated secondary antibodies (1:10,000, Millipore, AP307P), respectively. Blots were revealed by chemiluminescence (ECL Prime Western Blotting Detection Reagent, Amersham Biosciences, RPN 2232) using Hyperfilm substrates (Amersham Biosciences, RPN 1674K). Relative protein expression was determined using the densitometry tool of Adobe Photoshop CS5 software. The bands were evaluated in grayscale, subtracting the background value, and the TNF-α/β-tubulin ratio was calculated for each sample.

Statistical analysis

Mechanical thresholds measured with the von Frey test provide discrete values corresponding to filaments' values, thus limiting the relevance of classical parametric multi-factor analysis of variance (ANOVA). An ANOVA-type multiple-factor nonparametric methodology for longitudinal data, which can take into account both within and between factors, has recently been developed[38] as a package (nparLD) for R (version 3.2.1). We used the nparLD function to analyze the effects of time, side (left vs. right paw), sex (male vs. female), and of treatment (e.g. surgery and/or drug dose). The asymptotic ANOVA-type statistic (ATS) is provided as $ATS_{(d.f.)}$, with its adjusted degrees of freedom (d.f.) and p value. Multiple comparisons between groups at a given time point were performed with the two-sample Wilcoxon test, with the corresponding Bonferroni adjustment. The Wilcoxon test was also used for

comparison of the mechanical sensitivity thresholds between males and females. Immunoblotting experiments were analyzed with the nonparametric Kruskal–Wallis test, followed by multiple comparisons with the Wilcoxon test. The significance level was set at $p < 0.05$. Data were represented as mean ± SEM.

Results

Antiallodynic action of chronic oral pregabalin: Dose response

The mechanical sensitivity of the C57BL/6J mice was assessed using von Frey hairs. Although sham surgery did not influence mechanical thresholds (Figure 1(a) and (b)), cuff implantation induced an ipsilateral mechanical allodynia (Figure 1(a); surgery × time interaction, $ATS_{(2.9)} = 3.9$, $p < 0.005$ on postsurgery days 1–19). We did not observe any change in the nociceptive threshold of the left paw, contralateral to the cuff implantation; 19 days after surgery, we started treatment with different doses of pregabalin (300, 100, 50, or 5 μg/mL) or with vehicle solution (0.02% saccharin). Pregabalin treatment at doses 100 and 300 μg/mL alleviated the cuff-induced allodynia after about three days of treatment (Figure 1(a); group × time interaction, $ATS_{(13.9)} = 2.8$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" < "Cuff Pregabalin 100 μg/mL and Pregabalin 300 μg/mL" at $p < 0.05$ on postsurgery days 22–40). A partial antiallodynic effect was also present with the 50 μg/mL dose of pregabalin after eight days of treatment (Figure 1(a); multiple comparisons: "Cuff Vehicle" < "Cuff Pregabalin 50 μg/mL" < "Sham Vehicle" at $p < 0.05$ on postsurgery days 27–40). Treatments at different doses did not affect the contralateral nociceptive thresholds (Figure 1(a)). The 5 μg/mL dose of pregabalin had no significant effect (Figure 1(a)).

Chronic oral treatment with pregabalin at 300 μg/mL suppressed cuff-induced allodynia (Figure 1(a)), but it did not affect mechanical thresholds of mice of the Sham group (Figure 1(b)).

The drinking bottles were regularly weighed during the experiment. Considering the volume of solution drank by the mice per 24 h, the 5 μg/mL solution was equivalent to 0.78 ± 0.05 mg/kg/day, the 50 μg/mL solution was equivalent to 8.09 ± 0.38 mg/kg/day, the 100 μg/mL solution was equivalent to 15.64 ± 0.65 mg/kg/day, and the 300 μg/mL solution was equivalent to 44.63 ± 1.39 mg/kg/day (Figure 1(c)). These amounts were in fact mostly taken over the 12 h night period, period during which mice usually drink.

Body weights of mice treated chronically with different doses of pregabalin or vehicle were also assessed throughout the experiment. Cuff animals showed a difference in weight gain in the days following the surgery compared to Sham animals. This difference persisted in Cuff mice treated with vehicle or pregabalin at doses of 5 and 50 μg/mL. Pregabalin treatment at doses of 100 and 300 μg/mL, which relieved neuropathic allodynia, reversed this deficit in weight gain (Figure 1(d); group × time interaction, $ATS_{(11.2)} = 6.2$, $p < 0.001$; multiple comparisons: "Cuff Vehicle, Pregabalin 5 μg/mL and Pregabalin 50 μg/mL" < "Sham Vehicle" at $p < 0.05$ on postsurgery days 7–40, "Cuff Pregabalin 100 μg/mL and Pregabalin 300 μg/mL" < "Sham Vehicle" at $p < 0.01$ on postsurgery days 7–19 and "Cuff Vehicle" < "Cuff Pregabalin 100 μg/mL and Pregabalin 300 μg/mL" at $p < 0.01$ on postsurgery days 25–40).

Response to pregabalin: Male/female comparison in wild-type mice

Mechanical sensitivity thresholds of female mice were significantly lower than in males (baseline threshold values of paws are equal to 4.67 g ± 0.19 for males and 3.28 g ± 0.13 for females, male vs. female: $W = 79.5$, $p < 0.001$). Both male and female mice developed mechanical allodynia after cuff implantation and pregabalin treatment suppressed the cuff-induced allodynia in both sexes (Figure 2(a); Male mice: group × time interaction, $ATS_{(6.1)} = 7.5$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" < "Sham Vehicle" at $p < 0.05$ on treatment days 0–12 and "Cuff Vehicle" < "Cuff Pregabalin 300 μg/mL" at $p < 0.05$ on treatment days 9–12; Female mice: group × time interaction, $ATS_{(5.9)} = 5.1$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" < "Sham Vehicle" at $p < 0.05$ on treatment days 0–12 and "Cuff Vehicle" < "Cuff Pregabalin 300 μg/mL" at $p < 0.05$ on treatment days 9–12).

Chronic oral pregabalin treatment in opioid receptor deficient mice

The MOP, DOP, or KOP receptors-deficient mice displayed baselines for mechanical sensitivity that were similar to the wild-type littermates (Figure 2(b)). We controlled in our facilities that morphine has no more action in MOP-deficient mice.[36] Two weeks after surgery, we started the oral treatment with either pregabalin (300 μg/mL) or vehicle (0.02% saccharin) solutions. Pregabalin treatment alleviated cuff-induced allodynia in wild-type mice (Figure 2(b); group × time interaction, $ATS_{(6.9)} = 13.1$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" < "Cuff Pregabalin" at $p < 0.05$ on treatment days 9–12). The same antiallodynic effect was also present in MOP receptors (Figure 2(c); group × time interaction, $ATS_{(5.2)} = 10.4$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" < "Cuff Pregabalin" at $p < 0.05$ on treatment days 9–12), DOP receptors (Figure 2(c); group × time interaction, $ATS_{(7.1)} = 8.8$, $p < 0.001$;

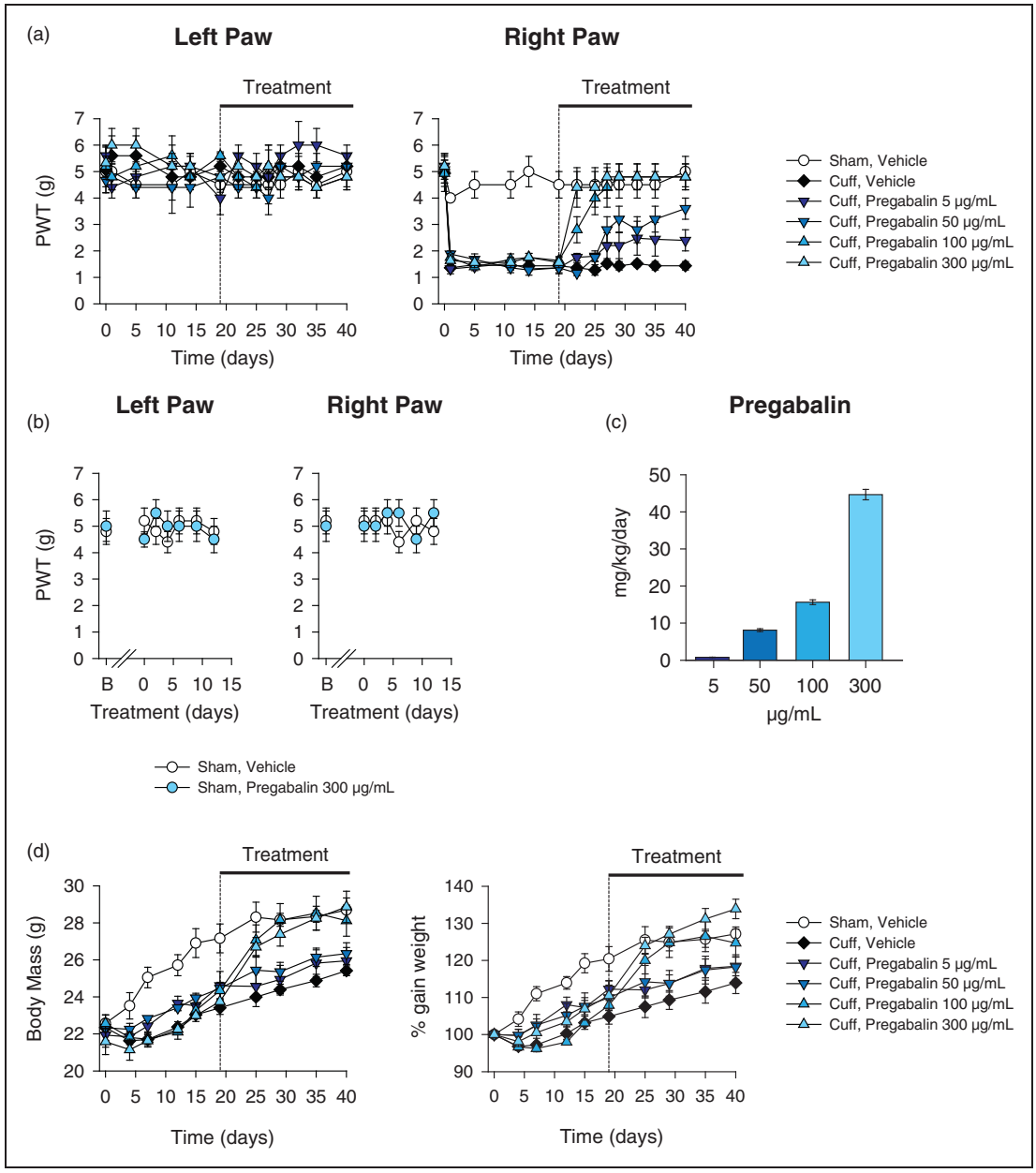

Figure 1. Chronic pregabalin treatment. (a) Two weeks after unilateral cuff insertion around the right sciatic nerve, chronic oral treatment with pregabalin started and lasted three weeks. The animals ($n = 5$ per each group) freely drink pregabalin (5, 50, 100, or 300 µg/mL) with 0.02% saccharin, or vehicle composed of 0.02% saccharin in water, as sole source of fluid. Mechanical PWT were evaluated at indicated time points using von Frey filaments. Vehicle treatment did not affect mechanical sensitivity of either Sham or Cuff mice. Pregabalin treatment was ineffective at dose 5 µg/mL, partially effective at dose 50 µg/mL, and reversed the cuff-induced allodynia at doses 100 and 300 µg/mL. (b) Pregabalin treatment at dose 300 µg/mL had no effect *per se* on sham-operated mice. (c) Histogram showing the equivalence between µg/mL and mg/kg/day of the different doses. (d) Time course of changes in the body weight of the animals throughout the experiment. Data are expressed as mean ± SEM.

multiple comparisons: "Cuff Vehicle" < "Cuff Pregabalin" at $p < 0.05$ on treatment days 9–12), and KOP receptors-deficient mice (Figure 2(c); group × time interaction, $ATS_{(5.5)} = 8.4$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" < "Cuff Pregabalin" at $p < 0.05$ on treatment days 9–12). Thus, pregabalin suppressed cuff-induced allodynia independently of the presence or no of the opioid receptors.

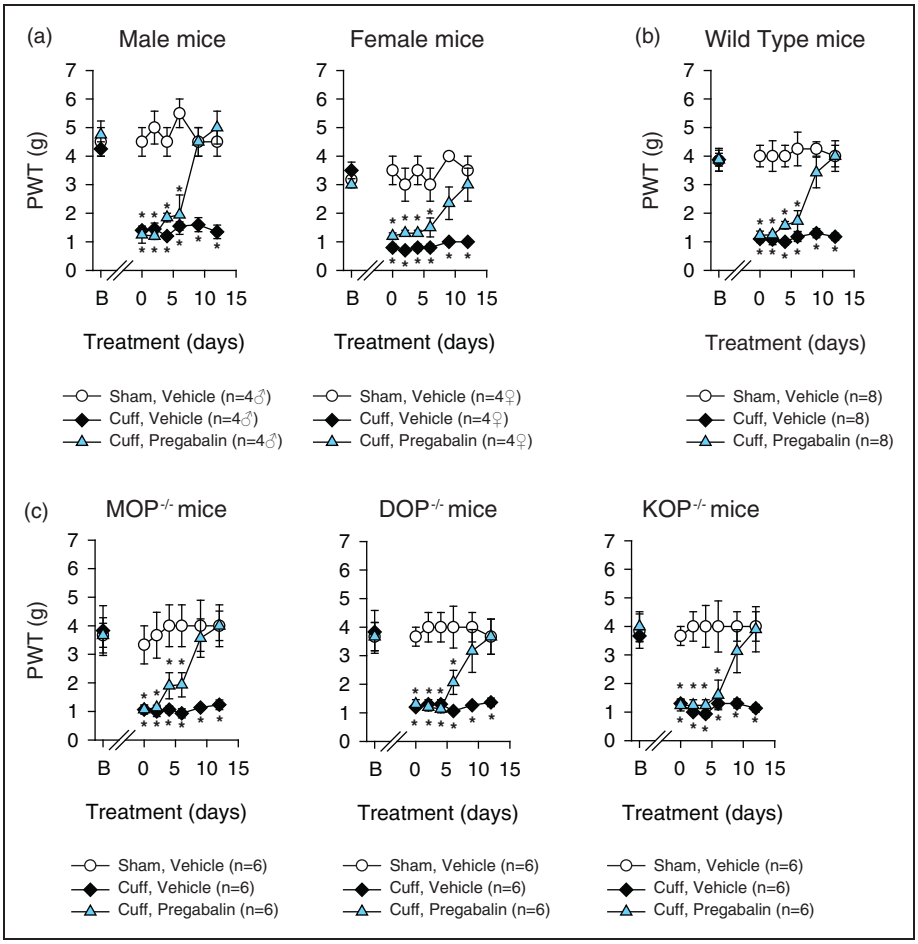

Figure 2. Effect of chronic oral pregabalin in opioid receptor deficient mice. Pregabalin treatment (300 μg/mL i.e 44.63 mg/kg/day in the drinking water, with 0.02% saccharin) or control treatment (0.02% saccharin) started two weeks following surgery and lasted 12 days. Mechanical allodynia was tested using von Frey hairs. (a) The mechanical sensitivity threshold (PWT) of female mice is lower than that of male mice. However, both sexes developed mechanical allodynia similarly and pregabalin was effective in reversing the cuff-induced allodynia in both male and female mice. Males and females were then pooled in each experimental group. (b) Chronic pregabalin treatment abolishes the ipsilateral cuff-induced allodynia in wild type mice, as well as in MOP, DOP, or KOP receptors-deficient mice (c). (Data are pooled from three independents experiments, each final group includes the same number of male and female mice, $^{*}p < 0.05$ as compared with Sham-operated control group drinking vehicle). Data are expressed as mean ± SEM.

Naloxone effect on long lasting pregabalin treatment

We tested the consequence of an acute injection of the opioid receptor antagonist naloxone (1 mg/kg, s.c.) on the antiallodynic action of pregabalin in C57BL/6 J male mice. After 10 days of oral treatment with pregabalin or vehicle (Figure 3(a); group × time interaction, $ATS_{(11.1)} = 9.3$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" < "Cuff Pregabalin" at $p < 0.005$ on postsurgery days 19 to 24 and "Cuff Pregabalin" = ("Sham Pregabalin" or "Sham Vehicle") at $p = 1.0$ on postsurgery days 22 and 24), acute injection of naloxone did not suppress the antiallodynic effect of chronic pregabalin treatment (Figure 3(c)). We also observed that naloxone per se had no effect in mice with Sham surgery or in mice that received vehicle alone (Figure 3(b)).

Transitory relief of neuropathic allodynia by acute pregabalin

In wild-type mice, an acute injection of pregabalin at a high dose (30 mg/kg, i.p.) had a transitory antiallodynic effect in Cuff mice, without affecting Sham animals (Figure 4; group × time interaction, $ATS_{(2.7)} = 12.3$, $p < 0.001$; multiple comparisons: "Cuff Pregabalin" = "Sham Pregabalin" at $p > 0.7$ on post-administration time 60 min and "Cuff Pregabalin" < "Sham Pregabalin" at $p < 0.001$ on post-administration time

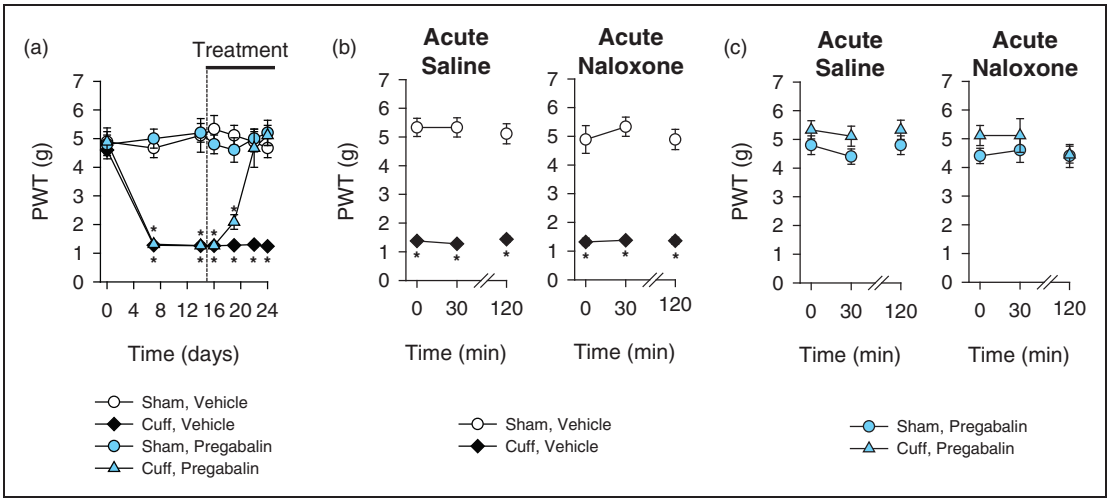

Figure 3. Acute opioid receptor antagonist in chronic pregabalin treatment. (a) Two weeks after unilateral cuff insertion, the oral treatment with pregabalin, or vehicle control started. Mechanical threshold of hindpaw withdrawal (PWT) was evaluated using von Frey filaments. Pregabalin treatment suppressed the cuff-induced alloynia. (b, c) After at least 10 days of pregabalin (300 μg/mL i.e 44.63 mg/kg/day, 0.02% saccharin) or vehicle treatment, the animals received an injection of the opioid receptor antagonist naloxone (1 mg/kg, s.c.) or the control saline solution. Mechanical threshold for hindpaw withdrawal was measured before 30 and 120 minutes after injection. No effect of naloxone or saline was seen in Sham mice or in pregabalin-treated neuropathic animals ($n = 9$–10, $^*p < 0.005$ compared to the Sham-operated control group). Data are expressed as mean ± SEM.

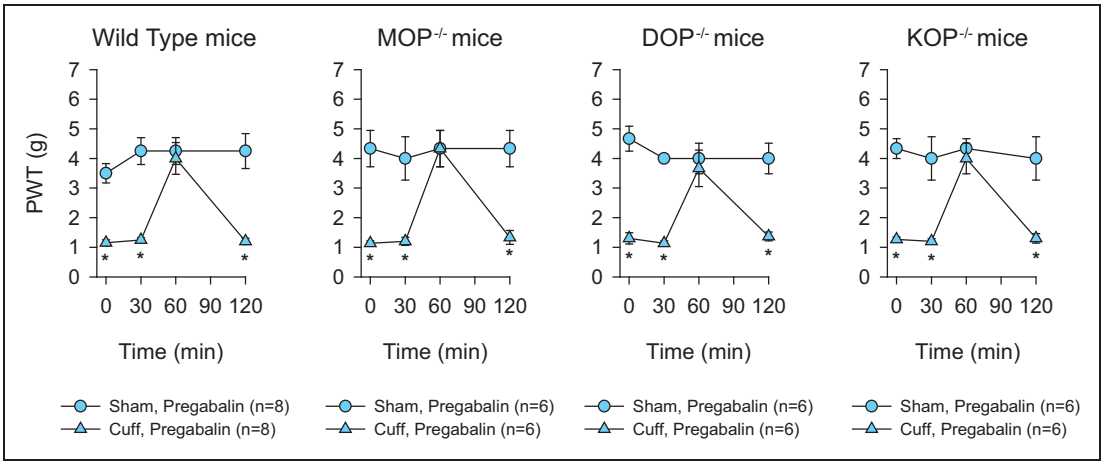

Figure 4. Effect of acute pregabalin in opioid receptor-deficient mice. Two weeks after cuff implantation, the animals received an acute injection of saline (i.p.) or of pregabalin (30 mg/kg, i.p.). Nociceptive mechanical threshold (PWT) was tested before (0 min) 30, 60, and 120 min after these acute injections. Acute pregabalin had a transitory antiallodynic effect in wild type Cuff mice without affecting Sham animals. Similar results were obtained in MOP, DOP, and KOP receptors-deficient mice. (Number of animals are given between brackets, data are pooled from three independent experiments, each final group includes the same number of male and female mice, $^*p < 0.005$ compared to Sham-operated controls receiving pregabalin.) Data are expressed as mean ± SEM.

0, 30, and 120 min). The same transitory effect was also present in MOP receptors (Figure 4; group × time interaction, $ATS_{(1,.6)} = 11.1$, $p < 0.001$; multiple comparisons: "Cuff Pregabalin" = "Sham Pregabalin" at $p = 1.0$ on post-administration time 60 min and "Cuff Pregabalin" < "Sham Pregabalin" at $p < 0.01$ on post-administration time 0, 30, and 120 min), DOP receptors (Figure 4; group × time interaction, $ATS_{(2.2)} = 12.7$, $p < 0.001$; multiple comparisons: "Cuff Pregabalin" = "Sham Pregabalin" at $p > 0.7$ on post-administration time 60 min and "Cuff Pregabalin" < "Sham Pregabalin" at $p < 0.01$ on post-administration time 0, 30, and 120 min),

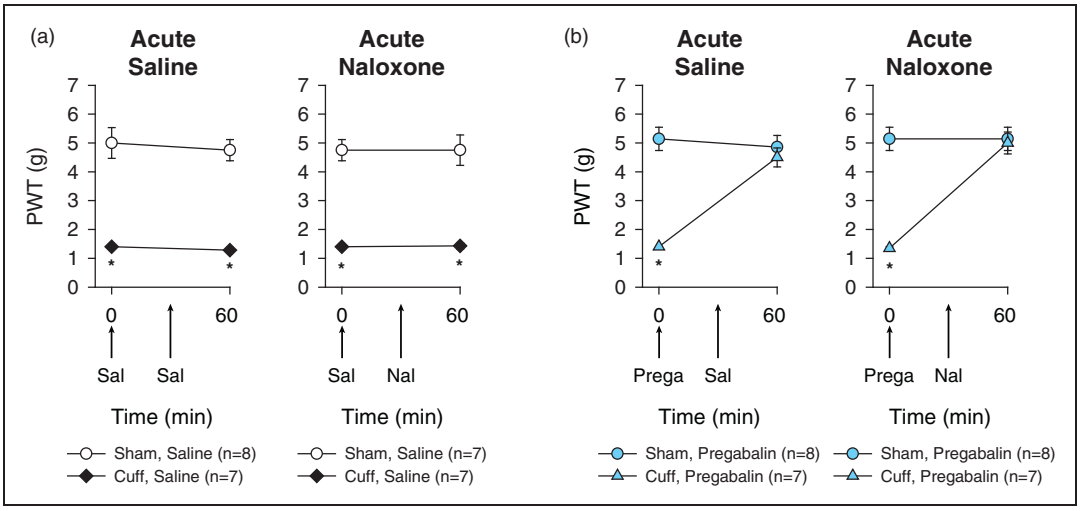

Figure 5. Acute opioid receptor antagonist in acute pregabalin treatment. Two weeks after unilateral cuff surgery, mice received an injection of pregabalin (30 mg/kg, i.p.) or saline control; 30 min later, they received an injection of the opioid receptor antagonist naloxone (1 mg/kg, s.c.) or control saline solution. Mechanical threshold for the right hindpaw (PWT) was measured before the first injection and 30 min after the second injection. (a) Naloxone and saline had no effect in Sham mice and in Cuff mice that received control treatment ($n = 7$–8, $^*p < 0.005$ compared to the Sham-operated control group). (b) Naloxone and saline had no effect in Sham mice and in Cuff mice that received pregabalin treatment (30 mg/kg, i.p.) ($n = 7$–8, $^*p < 0.005$ compared to the Sham-operated control group). Data are expressed as mean ± SEM.

and KOP receptors-deficient mice (Figure 4; group-× time interaction, $ATS_{(2.3)} = 10.5$, $p < 0.001$; multiple comparisons: "Cuff Pregabalin" = "Sham Pregabalin" at $p > 0.6$ on post-administration time 60 min and "Cuff Pregabalin" < "Sham Pregabalin" at $p < 0.01$ on post-administration time 0, 30, and 120 min). These transitory antiallodynic effects disappeared 120 min after injection of pregabalin.

Naloxone effect on acute pregabalin treatment

Naloxone (1 mg/kg) did not suppress the transitory antiallodynic action of acute pregabalin administration (Figure 5(a); group interaction, $ATS_{(1.0)} = 181.7$, $p < 0.001$; multiple comparisons: "Cuff Saline" < "Sham Saline" at $p < 0.001$ for acute saline administration and at $p < 0.005$ for acute naloxone administration) (Figure 5(b), acute saline; group × time interaction, $ATS_{(1.0)} = 12.7$, $p < 0.001$; multiple comparisons: "Cuff Pregabalin" < "Sham Pregabalin" at $p < 0.001$ preinjection and "Cuff Pregabalin" = "Sham Pregabalin" at $p > 0.5$ post-injection; Acute Naloxone; group × time interaction, $ATS_{(1.0)} = 13.7$, $p < 0.001$; multiple comparisons: "Cuff Pregabalin" < "Sham Pregabalin" at $p < 0.001$ preinjection and "Cuff Pregabalin" = "Sham Pregabalin" at $p > 0.8$ postinjection).

Long-term pregabalin has an anti-TNF-α action

Using Western blot, we observed increased levels of the membrane-bound form of TNF-α (mTNF-α) in the lumbar DRG of C57BL/6J Cuff mice at four weeks post-injury. The long-term treatment with pregabalin reversed this increase in mTNF-α. (Figure 6; $H_{(2.0)} = 16.2$, $p < 0.001$; multiple comparisons: "Cuff Vehicle" > ("Cuff Pregabalin" or "Sham Vehicle") at $p < 0.005$).

Discussion

In the present work, we studied the role of opioid receptors in both the long-term and the acute transitory antiallodynic action of systemic pregabalin in a model of neuropathic pain. In both cases, we show that the endogenous opioid system is not necessary for this action. We also show that a long-term pregabalin treatment suppresses the DRG TNF-α overexpression that accompanies neuropathic pain.

Clinically, first line pharmacological treatments to relieve neuropathic pain include anticonvulsants and antidepressants. Gabapentinoid anticonvulsants, which target the VDCCs α2δ-1 subunit, have proved to be effective in a number of neuropathic pain conditions.[3,39] Similarly to many reports in various animal models,[6,20,40,41] we showed that pregabalin has a short-term transitory antiallodynic action after an acute administration; however, this effect cannot be considered as representative of the main clinical therapeutic effect since the mechanical allodynia reappears within 2 h following the injection. Interestingly, the benefit of pregabalin treatment is sustained after three days of oral administration, which is in agreement with other results

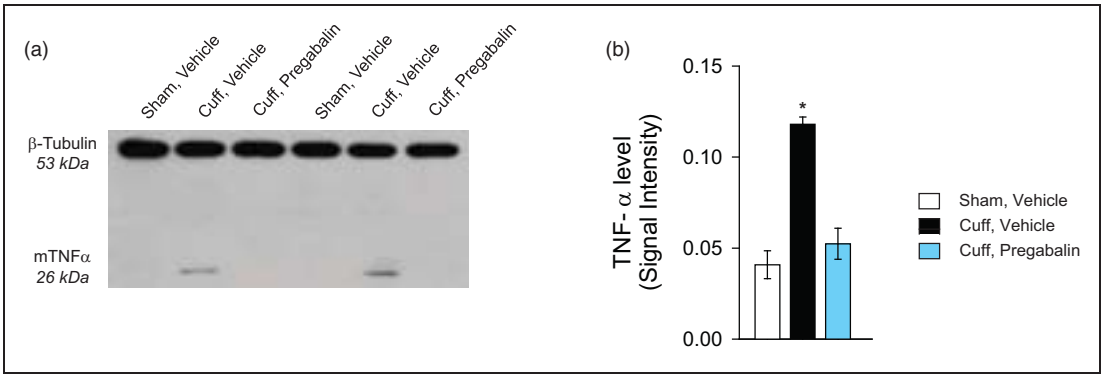

Figure 6. Long-term pregabalin displays an anti-TNF-α action on lumbar dorsal root ganglia of neuropathic mice. (a) Representative picture of Western blot illustrating the increased TNF-α levels in DRG of Cuff mice four to five weeks after induction of the neuropathy, and the anti-TNF-α action of the long-term pregabalin treatment (300 μg/mL i.e. 44.63 mg/kg/day). (b) Histogram presenting the Western blot analysis ($n = 8$ per group, $^*p < 0.005$ vs. Sham-vehicle). Data are expressed as mean ± SEM.

obtained with systemic delivery of another gabapentinoid, gabapentin,[20,42] or in other neuropathic pain models.[24,43] This sustained action may more likely be representative of the clinical use and action of gabapentinoids in neuropathic pain.[44,45]

Critical aspects of mechanism(s) by which gabapentinoids alleviate neuropathic pain is (are) now well described. Gabapentinoids inhibit calcium currents through direct interaction with the α2δ-1 subunit, thus decreasing excitatory transmitter release and spinal sensitization.[8,46] This target subunit is upregulated in the dorsal horn of the spinal cord and in DRG neurons in several models of neuropathic pain and this increase in α2δ-1 correlates with the onset of allodynia.[47] Furthermore, experiments performed in transgenic mice overexpressing the α2δ-1 subunit showed enhanced calcium currents recorded in DRG neurons, as well as nociceptive behavior characterized by hyperalgesia in the absence of nerve damage.[48] In contrast, α2δ-1 deficient mice display reduced DRG calcium currents, have lower baseline mechanical sensitivity, and show delayed mechanical hypersensitivity after partial sciatic nerve ligation.[49] In DRG neurons, α2δ-1 upregulation recruits mitochondrial Ca^{2+} to prolong intracellular Ca^{2+} signals evoked by depolarization.[50] This mechanism may contribute to the aberrant neurotransmission observed in neuropathic pain. Pregabalin antiallodynic effect is associated with decreased trafficking of the α2δ-1 subunit to presynaptic terminals of DRG neurons;[8,46] and within the dorsal horn, gabapentinoids also decrease the amplitude of excitatory postsynaptic currents.[51]

In addition to these actions, two studies suggested that gabapentinoids may also recruit the endogenous opioid system,[18,19] which is well known for playing a crucial role in the control of nociception and pain.[10,11,52] Indeed, the opioid antagonist naloxone reversed the acute antinociceptive activity of a high dose of pregabalin in naive mice.[18] Another study also showed an effect of naltrexone on the acute action of gabapentin in a model of orofacial inflammatory pain.[19] These recent data differ from previous studies on gabapentinoid drugs, which mostly reported naloxone to be ineffective in blocking gabapentinoid-induced analgesia in different pain models.[20–22] However, most of these studies were not done in models of neuropathic pain, which is the clinical pain condition for which gabapentinoids have legal authorization for prescription in various countries. Beside pharmacological approach, the present study used genetic deletion of opioid receptors for the first time, which further clarifies the involvement of the opioid system in both acute and chronic antiallodynic action of pregabalin in neuropathic pain. We demonstrate that neither acute nor long-term antiallodynic effect of pregabalin requires the presence of opioid receptors. Both our results and previous studies[20–22] refute the involvement of the opioid system in the antiallodynic action of pregabalin in neuropathic pain, which does not exclude a possible involvement of these receptors in gabapentinoid action on other types of pain.

The opioid system via MOP, DOP, and KOP receptors plays a crucial role in the inhibitory controls of pain[10,52,53] and also participates in the therapeutic action of various pain killers. Thus, MOP receptors are the primary molecular target for the analgesic action of opioids such as morphine, codeine, fentanyl, or tramadol.[10,54,55] Indirectly, the opioid system is also necessary for the antiallodynic action of tricyclic antidepressant drugs, which requires DOP receptors, but not MOP or KOP receptors.[11,36,56] Our results strengthen the idea that antidepressant and anticonvulsant treatments alleviate neuropathic pain through independent mechanisms.

These mechanistic differences may be in favor of combination pharmacotherapy for the management of neuropathic pain using both gabapentinoids and antidepressants,[57,58] although the benefit of such a combination is still controversial,[3,59] or using both gabapentinoids and opioid drugs.[14–17]

In the last decade, there has been an increasing number of studies which now provide compelling evidence that neuropathic pain pathogenesis is not simply confined to changes in the activity of neuronal systems, but that it also involves interactions between neurons, immune cells, and glial cells, including the involvement of inflammatory cytokines and chemokines.[25,60] Indeed, peripheral nerve injury recruits the immune system at various anatomical locations, including the lesion site, DRG, spinal cord, and supraspinal sites associated with pain pathways.[25] Pro-inflammatory cytokines produced after nerve injury could participate to the initiation and maintenance of neuropathic pain. Among these cytokines, TNF-α has the ability to also favor production of other cytokines.[28] The direct anti-TNF-α drugs infliximab and etanercept are clinically used to treat autoimmune diseases,[61] and these drugs have been shown to have some action on neuropathic pain symptoms both in animal models and in humans.[26,30,62–64] In particular, infliximab and etanercept can relieve neuropathic allodynia in the model of neuropathic pain used for the present study.[26] Our results show that pregabalin can display an indirect anti-TNF-α action, as seen on DRG from mice with neuropathic pain. This result is in agreement with previous reports on gabapentin suggesting an indirect action of this drug on cytokines.[16,24] Thus, it has been proposed that gabapentin could upregulate the expression of the anti-inflammatory cytokine IL-10 in the spinal cord, leading to the inhibition of the expression of pro-inflammatory cytokines, TNF-α, but also IL-1β and IL-6.[16,24]

Conclusions

This study demonstrates that none of the three opioid receptors is necessary for the antiallodynic action of acute or chronic pregabalin in a neuropathic pain context. Moreover, long-term pregabalin treatment decreases TNF-α in DRG. Further studies will be needed to elucidate the mechanism by which the direct action of pregabalin on the neuronal VDCCs α2δ-1 subunit may downregulate DRG TNF-α expression, which is mostly produced by non-neuronal cells. While the direct action of pregabalin on its target provides an explanation for acute pregabalin action at high dose, the sustained effect of prolonged treatment suggests the involvement of other downstream mechanisms the elucidation of which may provide new candidates for pharmacological targeting.

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Authors Contributions

ES and MB equally participated to this work. MK and IY did all surgeries. RAH and RAC performed dose responses. MK, LN, and XW performed behavioral tests on chronically treated opioid-receptor deficient mice. MK performed behavioral tests concerning naloxone and acute pregabalin. MK and DD performed the Western blot experiment. MB, MK, IY, and ES codesigned and supervised all experiments. MK collected and analyzed all data. MK and MB drafted the article. All authors revised the article prior to submission.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors declare that they have no competing interests.

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II. A dual mechanism for duloxetine relief of neuropathic allodynia.

Kremer M, Yalcin I, Goumon Y, Nexon L, Daniel D, Wurtz X, Megat S, Ceredig RA, Chavant V, Theroux JF, Lacaud A, Lelievre V, Turecki G, Lutz PE, Ernst C, Gilsbach R, Salvat E, Barrot M.

Résumé

La duloxétine, un antidépresseur inhibiteur sélectif de recapture de la sérotonine et de la noradrénaline, est l'un des traitements de référence des douleurs neuropathiques. Mieux comprendre son mécanisme d'action est crucial pour améliorer la tolérance et l'efficacité de ce traitement. Pour réaliser une recherche translationnelle nous disposons d'un modèle animal, validé chez la souris, reproduisant la douleur neuropathique et sa réponse aux traitements existants. En combinant l'utilisation de lignées transgéniques ainsi que des approches lésionnelles, pharmacologiques et moléculaires, nous avons identifié deux mécanismes indépendants par lesquels la duloxétine peut soulager la douleur neuropathique. A doses faibles à moyennes, un traitement chronique par la duloxétine soulage durablement la douleur neuropathique. Ce mécanisme fait intervenir la noradrénaline du système nerveux périphérique, qui agit via les β_2-AR des ganglions rachidiens. Les récepteurs DOP périphériques sont également impliqués. A doses plus fortes, la duloxétine exerce une action antalgique, transitoire et centrale, en recrutant la noradrénaline des voies descendantes inhibitrices qui agit alors via les α_{2A}-AR de la moelle épinière. Ce mécanisme implique les récepteurs MOP et DOP centraux. La pertinence préclinique de ces deux mécanismes a ensuite été étudiée en comparant les taux plasmatiques de duloxétine chez les patients et chez les animaux. En clinique, les taux plasmatiques se rapprochent de ceux obtenus chez l'animal sous traitement prolongé à faibles doses, suggérant l'intervention d'un mécanisme plutôt périphérique. Une analyse transcriptomique de ce mécanisme présumé périphérique montre qu'il met en jeu une inhibition de l'inflammation neurogène périphérique et notamment de la voie de signalisation TNFα-Nuclear Factor kappa B (NFκB, *facteur de transcription nucléaire kappa B*).

Introduction

La douleur neuropathique est secondaire à une lésion ou une maladie affectant le système somatosensoriel (Jensen et al., 2011). Elle n'est pas ou mal soulagée par les antalgiques usuels, tels que les anti-inflammatoires non stéroïdiens ou les opiacés (Baron, 2006). Les antidépresseurs tricycliques (amitriptyline, Laroxyl®), ainsi que les SSNRI (duloxétine, Cymbalta®), sont recommandés comme traitements de première intention de la douleur neuropathique. Leurs cibles primaires communes sont les sites de recapture de la noradrénaline et de la sérotonine, mais les molécules ciblant sélectivement la sérotonine ne sont pas ou peu efficaces, suggérant un rôle préférentiel du système noradrénergique (Attal et al., 2006; Saarto and Wiffen, 2007; Benbouzid et al., 2008a; Dharmshaktu et al., 2012; Bohren et al., 2013). Cependant les données précliniques qui essaient de faire la lumière sur ce mécanisme sont parfois confuses, voire contradictoires. Cette complexité pourrait être liée à l'intervention de divers acteurs cellulaires et moléculaires.

De nombreuses études ont montré que les antidépresseurs avaient une action antiallodynique et / ou antihyperalgique dans divers modèles animaux de douleurs neuropathiques (Mico et al., 2006; Benbouzid et al., 2008a; Choucair-Jaafar et al., 2014; *cf. p.31*). Mais il est à noter que la majorité de ces travaux ont évalué l'effet d'une administration unique, souvent à fortes doses, d'antidépresseurs. Or, chez l'homme, les antidépresseurs sont rarement utilisés pour soulager une douleur neuropathique aigüe et un traitement au long cours est nécessaire pour aboutir à un soulagement efficace. De plus, la dose nécessaire pour obtenir un effet antiallodynique et / ou antihyperalgique avec un traitement prolongé ne permet pas de produire une analgésie aigüe (Benbouzid et al., 2008a; Saika et al., 2009; Katsuyama et al., 2013; Kusuda et al., 2013; Le Cudennec and Castagne, 2014). Toutefois, il pourrait être postulé que le mécanisme par lequel l'antidépresseur agit en aigu pourrait être le même que celui par lequel il agit après traitement prolongé. Néanmoins cette hypothèse, de similarité d'action entre traitement aigu ou prolongé restait encore à explorer.

Pour progresser dans la compréhension du ou des mécanisme(s) d'action d'un traitement aigu ou prolongé par les antidépresseurs dans le soulagement de la douleur neuropathique, il est important d'identifier le ou les substrat(s) neuroanatomique(s) impliqué(s). Une des étapes clés est de déterminer la source de noradrénaline endogène

recrutée par les antidépresseurs. En effet, la noradrénaline peut être libérée au sein de structures supraspinales et spinales (Yoshimura and Furue, 2006; Pertovaara, 2013; Llorca-Torralba et al., 2016) par les projections issues principalement du locus cœruleus, ou au niveau périphérique par les efférences sympathiques qui forment des bourgeonnements dans les ganglions rachidiens suite à une lésion des nerfs périphériques (McLachlan et al., 1993; Ramer and Bisby, 1998). L'hypothèse la plus documentée dans la littérature concerne l'action de ces traitements sur les systèmes de modulation de la douleur et en particulier sur les contrôles inhibiteurs descendants noradrénergiques (Ardid and Guilbaud, 1992; Suzuki et al., 2008; Arsenault and Sawynok, 2009). Mais une étude récente a aussi mis en évidence l'existence d'un mécanisme périphérique lors d'un traitement prolongé (Bohren et al., 2013 *cf. p.16*). Cette dichotomie est également retrouvée en ce qui concerne les récepteurs adrénergiques impliqués. De nombreuses données de la littérature suggèrent un effet analgésique des antidépresseurs médié par les α_2-AR (Ozdogan et al., 2004; Cegielska-Perun et al., 2013; Hajhashemi et al., 2014). Toutefois, d'autres études mettent en évidence un mécanisme dépendant sélectivement des β_2-AR (Yalcin et al., 2009a; Yalcin et al., 2009b; Bohren et al., 2013; Choucair-Jaafar et al., 2014), ou des adrénocepteurs à la fois α et β (Ucel et al., 2015).

Si le système noradrénergique est important dans l'action des antidépresseurs sur la douleur neuropathique, la ou les cascade(s) d'effecteur(s) secondaire(s) reste(nt) à préciser. Diverses études ont suggéré un rôle du système opioïdergique. En effet, la naloxone, un antagoniste des récepteurs des opioïdes, bloque l'effet aigu ou prolongé des antidépresseurs dans divers modèles de douleur neuropathique (Ardid and Guilbaud, 1992; Marchand et al., 2003; Benbouzid et al., 2008a; Wattiez et al., 2011; Ucel et al., 2015), mais l'identité des récepteurs impliqués reste pour l'heure discutée (Schreiber et al., 1999; Marchand et al., 2003; Benbouzid et al., 2008b; Bohren et al., 2010; Choucair-Jaafar et al., 2014; Megat et al., 2015).

Le but de cette étude était de décrypter le(s) mécanisme(s) d'action(s) de deux procédures différentes, l'une qui mime un effet aigu, rapide de la duloxétine, et une seconde qui reproduit une action chronique de cet antidépresseur avec un délai thérapeutique d'environ une semaine. Nous avons voulu connaître quelle(s) étai(en)t la ou les sources de noradrénaline, périphérique ou centrale, recrutées, ainsi que le ou les adrénocepteur(s) impliqué(s). Nous avons également voulu savoir quel était le rôle des différents récepteurs

des opioïdes dans l'effet de ces deux traitements. Pour répondre à ces questions, nous avons utilisé des approches lésionnelles, pharmacologiques et moléculaires ainsi que des lignées d'animaux transgéniques. A cette étape, une dualité d'action de la duloxétine a pu être mise en évidence. A doses faibles à moyennes, un traitement chronique par la duloxétine soulage durablement la douleur neuropathique par un mécanisme périphérique alors qu'à doses plus fortes, cet antidépresseur exerce une action antalgique, transitoire et centrale. Pour répondre à la question cruciale du ou des mécanisme(s) d'action réellement mis en jeu chez le patient, nous avons alors comparé les taux plasmatiques de duloxétine chez l'homme et chez l'animal. Les résultats montrent que les concentrations plasmatiques de duloxétine chez l'homme se rapprochent de celles obtenues chez l'animal sous traitement prolongé, suggérant une action antalgique plutôt périphérique que centrale. Pour aller plus loin dans la compréhension de ce mécanisme présumé périphérique, nous avons également réalisé une analyse transcriptomique qui a révélé que la duloxétine en traitement chronique agit principalement comme inhibiteur de l'inflammation neurogène périphérique.

Dans le cadre de ce travail, j'ai personnellement effectué les expériences de dose-réponse pour la duloxétine et l'amitriptyline, l'ensemble des expériences sur l'effet aigu de la duloxétine, les lésions noradrénergiques, les expériences avec les antagonistes adrénergiques, ainsi que les injections de siRNA, les prélèvements de moelle lombaire et les extractions de protéines en vue de la Real-Time Polymerase Chain Reaction (RT-PCR, *réaction en chaîne polymérase en temps réel*), réalisée par le Dr. Vincent Lelièvre. Pour les dosages plasmatiques chez les animaux, j'ai recueilli l'ensemble des données comportementales ainsi que le sang des animaux correspondants. La spectrométrie de masse a été réalisée par le Dr. Yannick Goumon. Les données de transcriptomique ont été obtenues à partir d'ARN de ganglions rachidiens, préalablement prélevés par mes soins. Une collaboration avec le laboratoire du Dr. Ralf Gilsbach et la plateforme GeneCore de Freibourg a été réalisée pour l'occasion. L'analyse initiale de ces résultats a été faite au Canada par le laboratoire de Dr. Turecki. J'ai ensuite étudié et interprété les données notamment dans leur aspect fonctionnel. J'ai également réalisé les expériences de biologie moléculaire afin d'évaluer l'effet d'un traitement prolongé par les antidépresseurs sur les taux de TNFα dans les ganglions rachidiens. Les patients pour l'étude des taux plasmatiques de duloxétine ont été recrutés par le Dr. Eric Salvat au Centre du Traitement et d'Evaluation de la Douleur des

Hôpitaux Universitaires de Strasbourg. De plus, j'ai directement encadré les étudiants ou techniciens qui ont effectué le reste des expériences comportementales.

Methods

Study Design

The purpose of this study was to understand how duloxetine relieves neuropathic pain. Using mice, a mechanistic dissection of this mechanism was performed and compared to the action of a tricyclic antidepressant, amitriptyline. Results highlighted that two independent mechanisms can mediate the action of duloxetine. A co-clinical study was then conducted to compare the plasma levels of duloxetine in neuropathic pain patients and in the animal model. As the results highlighted the importance of the chronic mechanism of duloxetine, we further explored this mechanism by a transcriptomic analysis.

Animals

Experiments were performed using C57BL/6J mice (Charles River, L'Arbresle, France) with ages between 8 and 10 weeks at surgery time, or with male and female mice lacking β_2-AR, MOP, DOP or KOP receptors and their littermate controls. The generation of mice lacking MOP, DOP or KOP receptors, created in the laboratory of Brigitte Kieffer (IGBMC, Strasbourg, France), has been previously described (Matthes et al., 1996; Simonin et al., 1998; Filliol et al., 2000). Mice lacking β_2-AR were created in the laboratory of Brian Kobilka (Stanford University, Stanford, CA) and have been described previously (Chruscinski et al., 1999). Heterozygote mice were bred in our animal facilities, genotyping of the litters was done, and the experiments were conducted on adult male and female wild-type (WT) and knockout littermate mice weighing 20-30 g. The same number of males and females was used in each experimental group. As the WT animals have the same background and the same behavior, they were pooled to form the control groups. Mice were group-housed two to five per cage and kept under a 12 hour light/dark cycle with food and water *ad libitum*. All animal studies were performed in accordance with protocols approved by the "Comité d'Ethique en Matière d'Expérimentation Animale de Strasbourg" (CREMEAS, CEEA35). All animals received proper care in agreement with European guidelines (EU 2010/63). In pharmacological studies, mice were randomly assigned to experimental groups. The experimenter was always blind to both genotype and treatment. Although the effect size of genotype and drug effects were not predictable, sample sizes in this study are consistent with our previous experiences with the mice and procedures, and with the norm in the field.

Neuropathic pain model

Neuropathic pain was induced by cuffing the main branch of the right sciatic nerve (Benbouzid et al., 2008c; Yalcin et al., 2014; *cf. p.6*). Surgeries were performed under ketamine (68 mg/kg) / xylazine (10 mg/kg) intraperitoneal (i.p.) anesthesia (Centravet, Tadden, France). The common branch of the right sciatic nerve was exposed and a cuff of PE-20 polyethylene tubing (Harvard Apparatus, Les Ulis, France) of standardized length (2 mm) was unilaterally inserted around it (Cuff group). The shaved skin was closed using suture. Sham-operated mice underwent the same surgical procedure without implantation of the cuff (Sham group).

Measure of mechanical allodynia

Mechanical allodynia was tested using von Frey hairs and results were expressed in grams. Tests were done during the morning, starting at least two hours after lights on. Mice were placed in clear Plexiglas boxes (7 cm x 9 cm x 7 cm) on an elevated mesh screen. Calibrated von Frey filaments (Bioseb, Vitrolles, France) were applied to the plantar surface of each hindpaw until they just bent, in a series of ascending forces up to the mechanical threshold. Filaments were tested five times per paw and the paw withdrawal threshold (PWT) was defined as the lower of two consecutive filaments for which three or more withdrawals out of the five trials were observed (Benbouzid et al., 2008c; Bohren et al., 2010; Yalcin et al., 2014).

Treatment procedures

The long-term treatment with antidepressants began two weeks after the surgical procedure (cuff-implantation or sham-surgery). Duloxetine (Interchim, D4223, Montluçon, France) and amitriptyline (Sigma-Aldrich, St. Quentin Fallavier, France) were delivered *per os* (200, 150, 100 or 50 µg/mL), through the drinking water with *ad libitum* access as sole source of fluid. The drugs were dissolved in water with 0.2% saccharin to increase palatability, and control mice were given a solution of 0.2% saccharin in water (vehicle solution). For acute administration, duloxetine was dissolved in water with 0.2% saccharin and administered *per os* (15, 20 or 30 mg/kg, 10 mL/kg) with feeding needles (Cadence science®, 7921, Staunton, USA). Amitriptyline was dissolved in 0.9% NaCl and acutely administered intraperitoneally (15 mg/kg, 5 mL/kg). To inhibit the NFκB pathway, mice received two injections per day

(morning and evening) of pyrrolidine dithiocarbamate (PDTC, 20 mg/kg, 5 mL/kg, i.p., Sigma-Aldrich, St. Quentin Fallavier, France) dissolved in 0.9% NaCl solution that was also used for control injections.

Yohimbine (20 µg/mL; Sigma-Aldrich, St. Quentin Fallavier, France), an antagonist of α_2-AR, and propranolol (50 µg/mL; Sigma-Aldrich, St. Quentin Fallavier, France), a non-selective antagonist of β-AR, were delivered *per os* through the drinking water during five days. Mice received these antagonists two weeks after the beginning of the long-term treatment, in co-treatment with duloxetine or vehicle for chronic experiments; or for one week before acute administration of duloxetine for acute experiments. The injection of naloxone methiodide (NLX Meth, 5 mg/kg, s.c., Sigma-Aldrich, St. Quentin Fallavier, France), a non-selective antagonist of opioid receptors which does not cross the blood-brain barrier, was performed after twenty-five days of duloxetine or amitriptyline treatment; or 90 min after the acute administration of duloxetine. Long-term and acute treatment experiments were conducted on independent sets of mice. The injection of naloxone, a non-selective antagonist of opioid receptors (NLX, 1 mg/kg, s.c., Sigma-Aldrich, St. Quentin Fallavier, France) and of methylnaltrexone, a non-selective antagonist of opioid receptors that cannot cross the blood-brain barrier (MNTX, 10 mg/kg, s.c., Sigma-Aldrich, St. Quentin Fallavier, France), were done 90 min after the acute administration of duloxetine.

For cellular and molecular experiments, nervous tissues and/or blood were collected at selected time after acute administration, or after three weeks of chronic treatment.

Noradrenergic lesions

Chemical peripheral noradrenergic lesion was done two weeks before nerve injury, using guanethidine monosulfate (Sigma-Aldrich, St. Quentin Fallavier, France), with five daily intraperitoneal injections (30 mg/kg, 5 mL/kg). For chemical denervation of spinal noradrenergic transmission, mice were injected intrathecally, with the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA; 20 µg per mouse, in 5 µL of 0.9% NaCl solution containing 100 µg/mL of ascorbic acid) under ketamine (68 mg/kg) / xylazine (10 mg/kg) i.p. anesthesia. Briefly, for thoracic administration, a 27-gauge needle connected to a 50 µL Hamilton syringe was inserted at the level of the last thoracic vertebrae, just above the lower rib used as a mark. A control group of non-lesioned mice underwent the same nerve cuffing procedure as the lesioned groups.

Immunostaining

Five weeks post-surgery, nerve injured and sham mice were deeply anesthetized with pentobarbital and perfused intracardially with 4% paraformaldehyde in phosphate buffer 0.1M (10 mL/min). Lumbar dorsal root ganglia (L4, L5 and L6) and spinal cord were dissected, postfixed, cryoprotected, embedded in OCT compound (Sakura Finetek, Villeneuve d'Ascq, France), frozen and cut into 14 µm thick sections that were mounted on Superfrost®Plus slides (O. Kindler GmbH, Freiburg, Germany). To evaluate tyrosine hydroxylase (TH) expression, we used standardized procedures (Kaufling et al., 2010), incubating the sections with a sheep anti-TH antibody (1:1000, Millipore AB1542, France Millipore, Molsheim, France). The sections were then incubated with secondary Cy3-conjugated donkey antibodies (1:300, Jackson ImmunoResearch, 713-165-147, Newmarket, UK). Sections were washed, mounted with VECTASHIELD, and viewed under a Nikon E80i microscope with ×10 and ×20 objectives, and images acquired with MBF Bioscience camera CX9000 using Picture Frame acquisition software (MBF Bioscience, Magdeburg, Germany).

Tissue and blood collection for molecular analysis

In separate experiments, dorsal root ganglia and/or lumbar spinal cord were collected either after two weeks of chronic oral antidepressant treatment or 120 min after acute administration of duloxetine for acute experiments. Mice were killed by cervical dislocation, the back was dissected and a midline incision was done in the lumbar vertebrae to extract the L4, L5 and L6 dorsal root ganglia ipsilateral to the surgery and the lumbar spinal cord from L3 to L6. The 3 dorsal root ganglia were pooled per animal, all tissue were quickly frozen and stored at -80°C until protein or RNA extraction.

For mice plasma assays of duloxetine, an intracardiac blood collection was performed in mice under deep general anesthesia (ketamine (68 mg/kg) / xylazine (10 mg/kg), i.p.) by inserting a needle in the left ventricle. Blood was then collected and placed in blood collection tubes (BD Vacutainer® Héparine de Lithium, 4mL, 367526). The samples were then centrifuged (1500 x g, 20°C, 15 min) and the plasma was recovered and frozen at -80°C.

Plasmatic dosages of duloxetine

To compare the plasma levels of duloxetine in neuropathic pain patients and in the animal model, the plasma levels were measured in animals on chronic duloxetine treatment, or 1, 2, 5, 10 and 24 hours after acute administration.

This humans study was approved by the regional ethics committee and was performed in accordance with the Declaration of Helsinki and its subsequent amendments, good clinical practice and all applicable regulatory requirements. The primary purpose of this protocol was to evaluate the residual plasmatic concentration (ie before the morning dose of duloxetine) in patients relieved of at least 30% of their neuropathic pain with duloxetine treatment. The secondary purpose of this protocol was to measure the plasmatic peak concentration (ie 6 hours after the morning dose of duloxetine; Senthamil Selvan et al., 2007) in patients relieved of at least 30% of their neuropathic pain with duloxetine treatment. Others secondary purposes were to assess the degree of relief from the intensity of neuropathic pain with chronic duloxetine and to evaluate the sensation of improvement experienced by the patient. The intensity of pain reported by the patient was measured on a Digital Scale with 11 points (Dworkin et al., 2005). The sensation of improvement experienced by the patient was assessed on a Patient Global Impression of Change in 7 points scale (Dworkin et al., 2005). The patients' inclusion criteria were: (i) aged 28 to 75; (ii) relieved of their neuropathic pain by 60 mg of duloxetine treatment (with a differential of >30% in the intensity of neuropathic pain with numerical scale before and after long term treatment); (iii) affiliated to a social security system; (iv) have signed an informed consent. The exclusion criteria were: (i) concomitant treatment with fluvoxamine, flecainide, propafenone, metoprolol, risperidone, verapamil, omeprazole or modafinil; (ii) necessary information could not be given to the patients; (iii) patient under judicial protection, guardianship or curatorship. A total of 4 patients were included.

Plasma and tissue measurements

Duloxetine quantification in the plasma of humans and animals. 10 pmoL of deuterated duloxetine internal standard (Alsachim, Illkirch, France) were added to 200 µL of lithium-heparine plasma. Plasmas were acidified up to 2.5% v/v with formic acid (final volume of 800µL). After centrifugation (20000 x g, 20°C, 15 min) the supernatant was submitted to a solid phase extraction (SPE) using a positive pressure manifold (Thermo

Electron). OASIS HLB SPE-cartridges (1 cm^3, 30 mg, Waters, Guyancourt France) were first activated with 1 mL of acetonitrile (ACN) and then washed with 1 mL of H_2O / formic acid 0.1% (v/v). The samples were loaded and the SPE-cartridges were washed with 1 mL of H_2O/formic acid 0.1% followed by 1 mL of ACN 5% / formic acid 0.1% (v/v/v). The elution was performed with 500 µL of ACN 50% / H_2O (v/v). Finally, eluted fractions were dried under vacuum prior to MS analysis.

Catecholamine quantification in the lumbar spinal cord. Spinal cords were homogenized with a tissue mixer in 200 µL of 0.5 µM ascorbic acid. Homogenates were sonicated 10 s at 90 W and centrifuged (18000 x g, 4°C, 15 min). Supernatant was recovered and the concentration of proteins was determined (Protein Assay, Bio-Rad, Marnes-la-Coquette, France). 10 µL of the extract was derived with the AccQ-Tag Ultra Derivatization kit (Watters, Guyancourt, France) according to the manufacturer. Briefly, 10 µL of samples were mixed with 35 µL of borate buffer and 10 µL of internal standards (D4-dopamine, D4-serotonin, C6-noradrenaline; Sigma Aldrich and Alsachim). Then, 10 µL of AccQtag reagent were added and have been shacked 10 min at 55°C. After centrifugation (20000 x g, 20°C, 5 min), the supernatant was submitted to a LC-MS/MS analysis.

LC-MS/MS instrumentation and analytical conditions

LC-analyses were used to determine the presence of duloxetine or catecholamines using the selected reaction monitoring mode (SRM). Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA, USA) coupled with an Endura triple quadrupole mass spectrometer (Thermo Scientific). The system was controlled by Xcalibur v. 2.0 software (Thermo Scientific). Dried samples (spinal cord extracts) were suspendend in 100 µL of H_2O/formic acid 0.1% (v/v) and 10 µL of the solution were loaded into an Accucore RP-MS column (ref 17626-102130; 100 x 2.1 mm 2.6 µm, Thermo Electron) heated at 35°C. For derived samples, 10 µL of the derivatization mix was analysed. Elutions were performed by applying linear gradient of buffers A/B. Buffer A corresponded to H_2O 99.9% / formic acid 0.1% (v/v), whereas buffer B was ACN 99.9% / formic acid 0.1% (v/v). A linear gradient of 20-85% of solvent B at 400 µL/min over 2.5 min was applied followed by a washing step (0.5 min at 85% of solvent B) and an equilibration step (1 min of 20% buffer B).

The SRM mode was performed at 3500 V of liquid junction voltage and 292°C capillary temperature. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The

collision gas pressure of Q2 was set at 2 mTorr of argon. The selection of the monitored transitions and the optimization of the collision energy (CE) were preliminary and manually determined.

The transitions and the corresponding collision energies used for SRM were the following: m/z 298.25 → m/z 123.75 (CE = 42 eV), m/z 298.25 → m/z 155.24 (CE = 23 eV) and m/z 298.25 → m/z 183.17 (CE = 28 eV) for duloxetine; m/z 301.25 → m/z 123.75 (CE = 40 eV), m/z 301.25 → m/z 157.12 (CE = 7 eV) and 301.25 → m/z 183.05 (CE = 27 eV) for D4-duloxetine; m/z 324.25 → m/z 171.10 (CE = 33 eV) for AccQtag-dopamine; m/z 328.25 → m/z 171.10 (CE = 35 eV) for AccQtag-D4-dopamine; m/z 340.25 → m/z 171.25 (CE = 35 eV) for AccQtag-noradrenaline; m/z 346.25 → m/z 171.25 (CE = 27 eV) for AccQtag-C6-noradrenaline.

The identification of the compounds was based on precursor ion, selective fragment ions and retention times obtained for duloxetine or derived-catecholamine and the corresponding internal standard. Absolute quantifications of the compounds were done using the ratio of daughter ions response areas on the internal standards.

siRNA experiments

α_2-AR subtypes involved in acute effects of duloxetine have been identified using a siRNA-mediated knockdown of *adra2a* and *adra2c*. Knockdown of *adra2a* and *adra2c* mRNA have been performed using ACCELL siRNA (respectively Cat. #E-049576-00 and #E-043744-00) designed and validated by ThermoFisher/Dharmacon (Lafayette, CO, USA). A non-targeting siRNA (Cat. #D-001910-10-05) was used as a negative control. Stock solutions for the targets and scrambled siRNA were prepared at 100 µM in 1x siRNA Buffer (diluted from Dharmacon 5x siRNA Buffer Cat. #B-002000-UB-100). Targets and scrambled negative siRNA were injected intrathecally (10 µL per mouse) under gaseous anesthesia (isoflurane 1.5-2%). Briefly, for lumbar intrathecal administration, a 27-gauge needle connected to a 50 µL Hamilton syringe was inserted between the L5 and L6 vertebrae, into sub-arachnoidal space. Placement of the needle was verified by the elicitation of tail flick movement. Time course study was done with quantitative RT-PCR to identify optimal time points for assessing knockdown.

Quantitative RT-PCR

Quantification of gene expression in dorsal root ganglia has been assessed with RT-PCR method. Total mRNA was extracted using RNAeasy quit (Quiagen) and the cDNA was generated using *iScript cDNA synthesis kit* from Biorad. Quantitative RT-PCR was performed on a Thermocycler Biorad MylQ (Biorad) using Biorad IQ Syber Green Supermix assay. PCR consisted in an initialization step at 95°C for 3 min and the amplification was realized for 40 cycles with denaturation at 95°C for 20 s, followed by annealing at 95°C for 20 s, an extension at 60°C for 20 s. All experiments were performed in duplicate sample deposits on the amplification plate. The relative abundance of each RNA target gene transcript was normalized besides an endogenous gene control hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glyceraldehyde phosphate dehydrogenase (GAPDH). Data were analyzed according to the standard curve method. Primers have been designed from Mus Musculus, using gene data bank and they are as follows: musADRA2afw, CCAAGCTGCAAGATCAACGA and musADRA2arev, TACGCACGTAGACCAGGATC; musADRA2bfw, CAGAAGAAGGGGACTAGTGGG and musADRA2brev, GGCAGTGTTTGGGGTTCACAT; musADRA2cfw, CCTACTGGTACTTCGGGCAA and musADRA2crev, CCAGTAGCGGTCCAGACTAA.

Immunoblot analysis

Total proteins were extracted in 150 µL lysis buffer (20 mM Tris pH 7.5; 150 mM NaCl; 10% glycerol; 1% NP-40; Protease Inhibitors Cocktail, Roche), quantitated with Bio-Rad Protein Assay Dye Reagent Concentrate and stored in Laemmli buffer (2% sodium dodecyl sulfate (SDS); 25% glycerol; 0.01% bromophenol blue; 0.125 M Tris pH 6.8). Ten µg of total protein from individual animals were resolved by 12% SDS-polyacrylamide gel electrophoresis under reducing conditions, and then transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon, transfer membranes, Millipore, IPVH00010). The blots were incubated for 1h in blocking agent (ECL kit, Amersham Biosciences), overnight with the antibodies specific for either TNFα (1:500, R&D Systems, AF-410-NA) or β-tubulin (1:50000, Abcam, ab108342), followed by rabbit anti-goat horseradish peroxidase (HRP)-conjugated secondary antibodies (1:12000, Abcam, ab97100) or goat anti-rabbit HRP-conjugated secondary antibodies (1:10000, Millipore, AP307P) respectively. Blots were revealed by chemiluminescence (ECL Prime Western Blotting Detection Reagent, Amersham Biosciences, RPN 2232) using

Hyperfilm substrates (Amersham Biosciences, RPN 1674K). Relative protein expression was determined using the densitometry tool of Adobe Photoshop CS5 software. The bands were evaluated in grayscale, subtracting the background value, and the TNFα/β-tubulin ratio was calculated for each sample.

RNAsequencing

Sample collection.

Total RNA was extracted from dorsal root ganglia tissues with the Qiagen RNeasy Mini Kit (Hilden, Germany), according to the instructions of the kit. The quality of samples was verified using a NanoDrop. The samples were then considered as high-quality when their absorbance ratio 260/280 was between 1.8 and 2. The stringent RNA quality was also controlled using Agilent 2100 bioanalyzer. High-quality RNA sample must show only two distinct peaks for 18S and 28S rRNAs, with a ratio of about 2. Each experimental group consisted of 4 animals.

Library preparation and sequencing.

Samples were prepared using Beckman Biomek FX laboratory automation workstation (robotics) and Illumina truseq chemistry, starting with 250 ng of total RNA. Next generation sequencing library preparation was performed on the Beckman Biomek FX using Illumina's TruSeq LT DNA adapters. For each sample, 1 µg of cDNA was used for library preparation. Three replicates were field. The samples were sheared on a Covaris S2 to ~300 bp according to the manufacturer's recommendation. Size selection was performed on the Biomek FX using the SPRIworks HT Reagent Kit. Each library was only tagged with one of Illumina's TruSeq LT DNA barcodes to allow library pooling for sequencing. Library quantization was performed using Invitrogen's Picogreen assay and the average library size was determined by running the libraries on a Bioanalyzer DNA 1000 chip (Agilent). Library concentration was validated by qPCR on a StepOne Plus real time thermocycler (Applied Biosystems), using qPCR primers, standards and reagents from Kapa Biosystems. Libraries were then sequenced on an Illumina MiSeq sequencer at a read length of 100 bp paired end.

Bioinformatic analyses.

For bioinformatic processing, we used : FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/links.html) and Trimmomatic (Bolger et al., 2014)

for adapter trimming; Bowtie2 for alignment; TopHat (Trapnell et al., 2009) for transcript counting; and DESeq2 (Love et al., 2014) for differential expression analysis, as previously described (Maussion et al., 2015; Farmer et al., 2016). *Alignment*. Following high-throughput sequencing, 100 bp paired-end reads were aligned to the mouse reference genome (mm10) using TopHat v2.1.0 (http://tophat.cbcb.umd.edu/) with a mate insert distance of 75 bp (-r) and library type fr-unstranded. Those reads that passed mapping quality of at least 50 were used for gene and transcript quantification. *Quantification*. Gene annotations used for quantification corresponded to Ensembl annotations (Ensembl release 75). For gene-level quantification we used HTSeq-count version 0.6.1p1 (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) to count fragments that overlapped genes identified through the aforementioned annotation (Anders et al., 2015). HTSeq-count was run with the intersection-nonempty mode for each sample and the results were combined to form a count matrix. *Differential expression analysis*. Genes with no mapped fragments were removed from the analysis. Furthermore, genes with low counts were removed by keeping only those which have at least 20 counts per sample in average. Differential expression analysis was performed using the DESeq2 generalized linear model to conduct pair-wise comparisons (e.g. saccharin-cuff/saccharin-sham, duloxetine-cuff/saccharin-cuff, etc…).

For our initial analysis, we used DAVID (DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases NIAID, NIH), ToppFun (ToppGene Suite, Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229) and Gene Set Enrichment Analysis (GSEA, v2.2.0, The Broad Institute/Massachusetts Institute of Technology) software.

Statistical analysis

Statistical tests were performed using STATISTICA 12 software (Statsoft, Tulsa, OK, USA). Results are presented as means ± standard error deviation (SEM) unless otherwise indicated. For the behavioral experiments, statistical analysis was performed using multifactor analysis of variance (ANOVA). The surgery procedure (Sham or Cuff) and the various treatments were taken as between-group factors. When needed, the time of measurement was taken as a within-subject factor. The Duncan test was used for post hoc comparisons. Immunoblotting

and quantitative RT PCR experiments were analyzed with the nonparametric Kruskal-Wallis test, followed by multiple comparisons with the Wilcoxon test. Student's t test was used for RNA sequencing data statistical analysis. The significance level was set at $p < 0.05$.

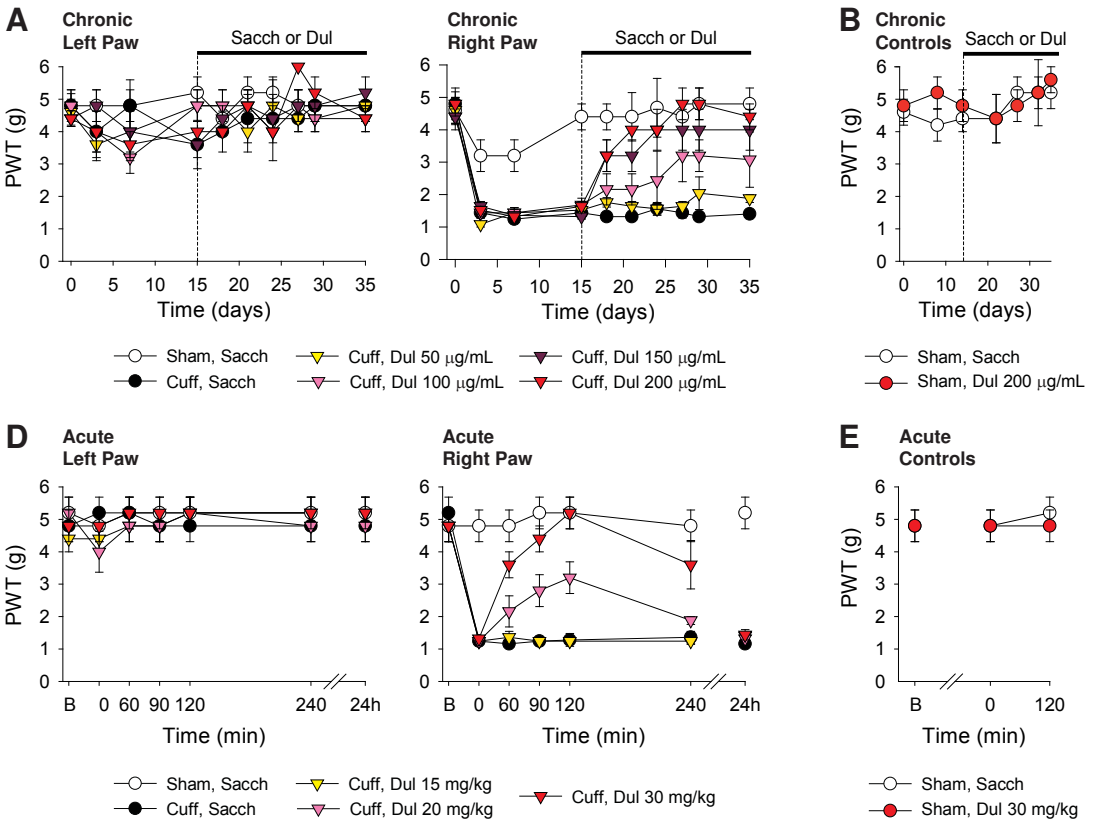


fig S1. Chronic and acute oral duloxetine treatment.
(**A**) The animals were administered duloxetine (50, 100, 150 or 200 µg/mL) in the drinking water with 0.2% saccharin as the sole source of fluid. The chronic 0.2% saccharin treatment did not affect the mechanical sensitivity of either Sham or Cuff mice. Chronic oral duloxetine treatment at dose 50 µg/mL did not reverse the allodynia. At doses 100, 150 or 200 µg/mL, long-term duloxetine treatment dose-dependently suppressed the cuff-induced allodynia. No effect of treatment at various doses is observed on the contralateral paw (n = 5 per group). (**B**) Chronic oral duloxetine (200 µg/mL) had no influence on the nociceptive threshold of Sham mice (n = 5 per group). (**C**) Histogram showing the equivalence between µg/mL and mg/kg/day. (**D**) Mice received an acute per os administration of duloxetine (15, 20 or 30 mg/kg) or water with saccharin 0.02%. Duloxetine produced an antiallodynic effect at the highest dose (30 mg/kg, n = 5 in each group). This effect was observed 60 to 240 minutes after acute administration. (**E**) The 0.2% saccharin treatment did not affect mechanical sensitivity of Sham mice (n = 5 per group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to Sham Saccharin Groups. B corresponds to the mechanical threshold sensitivity before surgery.

Results

Antiallodynic effects of prolonged vs. acute duloxetine in mice.

To mimic human neuropathy resulting from a trauma of peripheral nerves, we used chronic sciatic nerve cuffing in mice (Mosconi and Kruger, 1996; Benbouzid et al., 2008c; Yalcin et al., 2014). This model produced behavioral responses to non-noxious stimuli, called mechanical allodynia, after cuff implantation, although sham surgery did not influence mechanical thresholds (n = 5 mice/group, surgery x time interaction, $F_{9,216}$ = 2.9, p < 0.001; post-hoc: "Cuff" < "Sham" at p < 0.05 on post-surgery days 3 to 15) (**fig. S1A**). We observed no change in the nociceptive thresholds of the left paw, contralateral to the cuff implantation. Fifteen days after surgery, we started either long-term treatments with duloxetine (0, 50, 100, 150 or 200 µg/mL), or did an acute treatment with duloxetine at various high doses (0, 15, 20 or 30 mg/kg).

Long-term duloxetine treatment at doses 150 and 200 µg/mL alleviated cuff-induced allodynia after 10 days of treatment (n = 5 mice/group, surgery x time interaction, $F_{9,216}$ = 2.9, p < 0.001; post-hoc: "CuffSacch" < "CuffDul150" and "CuffDul200" at p < 0.001 on post-surgery days 24 to 35) (**fig. S1A**). A partial antiallodynic effect was also present at 100 µg/mL after 12 days of treatment (post-hoc: "CuffSacch" < "CuffDul100" at p < 0.05 on post-surgery days 27 to 35) (**fig. S1A**). The 50 µg/mL dose had no significant effect (**fig. S1A**). Treatments at different doses did not affect the contralateral nociceptive thresholds (**fig. S1A**). Chronic oral treatment with duloxetine at 200 µg/mL did not affect mechanical thresholds of mice of the Sham group (**fig. S1B**). The drinking bottles were regularly weighed during the experiment. Considering the volume of solution drank by the mice per 24 h, the 50 µg/mL solution was equivalent to 4.69 ± 0.12 mg/kg/day, the 100 µg/mL solution was equivalent to 8.58 ± 0.34 mg/kg/day, the 150 µg/mL solution was equivalent to 12.99 ± 0.76 mg/kg/day and the 200 µg/mL solution was equivalent to 20.09 ± 1.37 mg/kg/day (**fig. S1C**). These amounts were in fact mostly taken over the 12 h night period, period during which mice usually drink.

Acute *per os* administration of duloxetine dose-dependently increased the paw withdrawal threshold in mice with nerve injury (n = 5 mice/group, surgery x time interaction, $F_{6,120}$ = 5.5, p < 0.001; post-hoc: "CuffSacch" < "CuffDul20" at p < 0.001 on post-administration time 120 min and "CuffSacch" < "CuffDul30" at p < 0.001 on post-

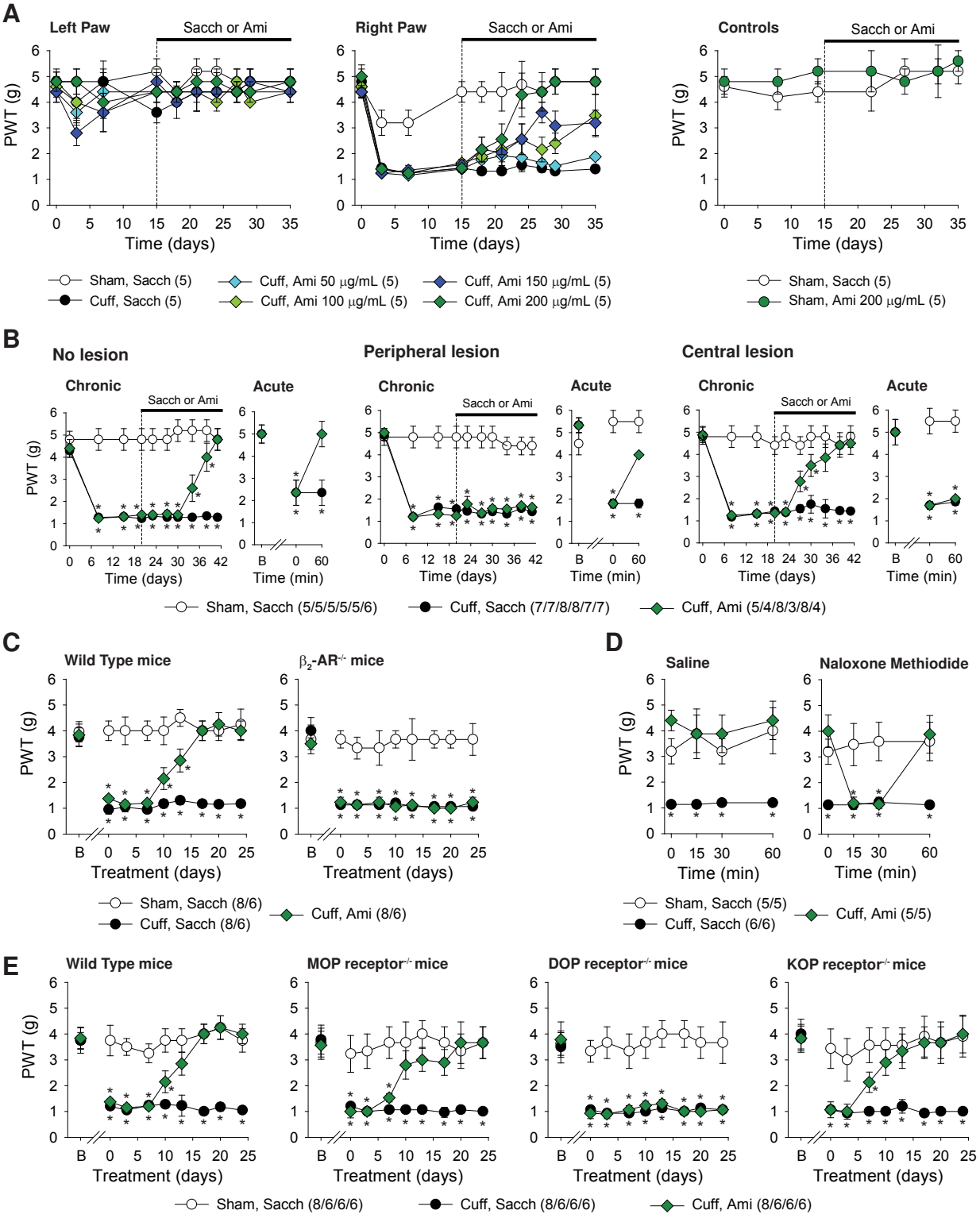


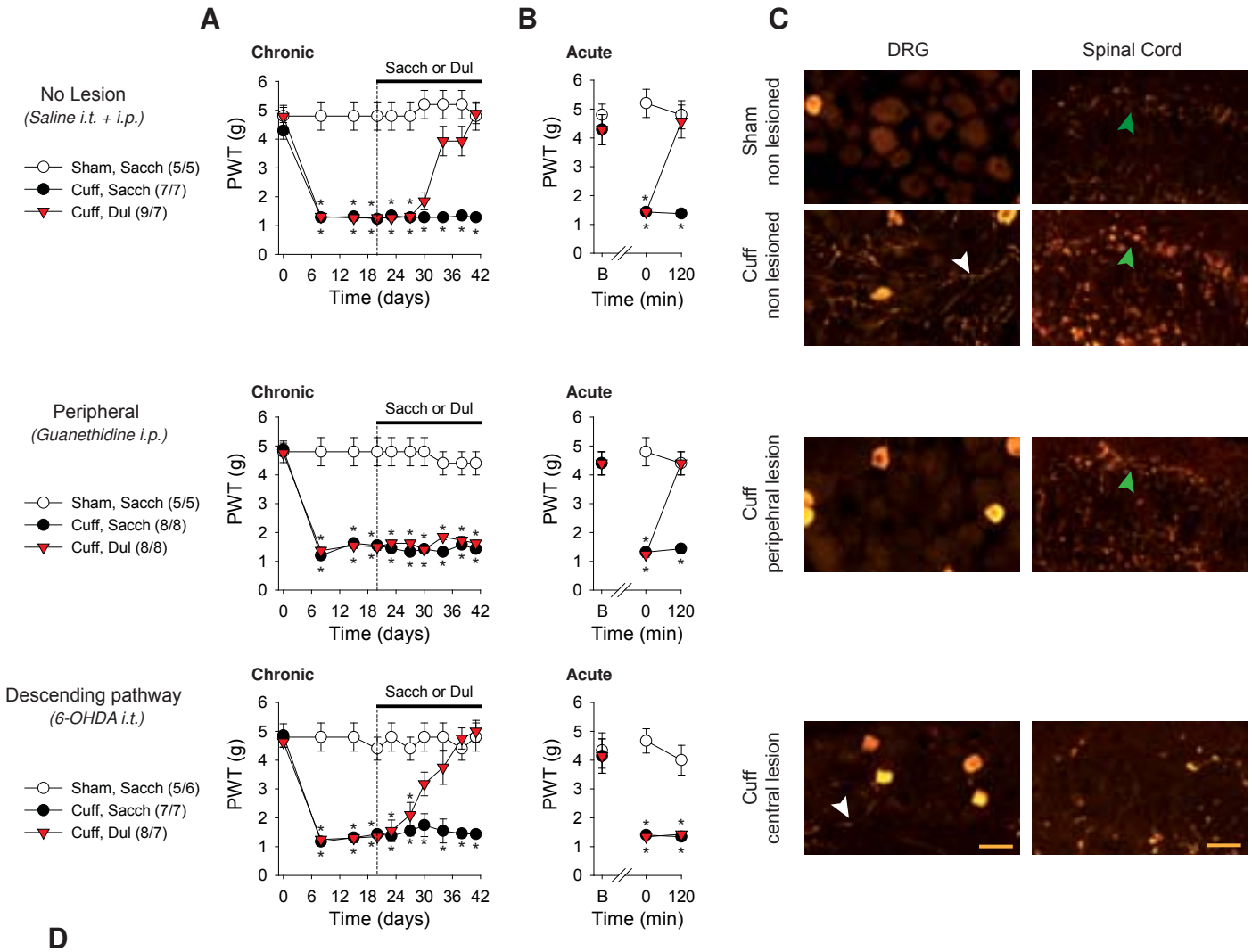
fig. S2. Mechanism of action of amitriptyline, a tricyclic antidepressant, in relieving mechanical allodynia in mice.
(**A**) The animals were administered amitritpyline (50, 100, 150 or 200 μg/mL) in the drinking water with 0.2% saccharin as the sole source of fluid. Chronic oral amitriptyline treatment at doses 100, 150 or 200 μg/mL dose-dependently suppressed the cuff-induced allodynia. No effect of treatment is observed on the contralateral paw or on the nociceptive threshold of Sham mice (n are given in bracket). (**B**) Guanethidine (peripheral lesion, 30 mg/kg, i.p.) and 6-OHDA (central lesion, 20 μg per mouse, i.t.) injections allowed lesioning the noradrenergic fibers. Chronic amitriptyline (200 μg/mL) exerted a delayed antiallodynic effect (left graph). The peripheral noradrenergic lesion (middle graph), but not the central (right graph), suppressed the long-term antiallodynic action of amitriptyline. Acutely, a high dose of amitriptyline (15 mg/kg) induced a rapid antiallodynic effect (left graph). This effect is prevented by a central noradrenergic lesion (right graph), but not by a peripheral lesion (middle graph) (Chronic/AcuteNo Lesion/Chronic/AcutePeripheralLesion/Chronic/AcuteCentralLesion n are given in bracket). (**C**) In wild type mice, chronic oral amitriptyline (200 μg/mL) suppressed mechanical allodynia but remained ineffective in β2-AR deficient mice (WT/β2-AR$^{-/-}$ n are given in bracket). (**D**) An acute injection of the opioid receptor antagonist, naloxone methiodide (5 mg/kg, s.c.) induced a relapse of allodynia in chronic amitriptyline-treated mice (Saline/NLX Meth n are given in bracket). (**E**) In wild-type mice, in MOP- and in KOP-deficient mice, chronic oral amitriptyline treatment suppressed the ipsilateral Cuff-induced allodynia, whereas it remained ineffective in DOP-deficient mice (WT/MOP$^{-/-}$/DOP$^{-/-}$/KOP$^{-/-}$ n are given in bracket). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to Sham Saccharin Groups. B corresponds to the mechanical threshold sensitivity before surgery.

administration times 60 to 240 min). Subsequent analysis showed that duloxetine at 30 mg/kg induced a significant antiallodynic effect from 60 to 240 min after administration, and 20 mg/kg from 90 to 120 min (**fig. S1D**). The 15 mg/kg dose of duloxetine had no significant effect (**fig. S1D**). Treatments at different doses did not affect the contralateral paw withdrawal thresholds (**fig. S1D**). Acute oral treatment with duloxetine at 30 mg/kg did not affect mechanical thresholds of mice of the Sham group (**fig. S1E**).

Similar results were obtained with the tricyclic antidepressant amitriptyline. Fifteen days after surgery, we started the long-term treatment with different doses (0, 50, 100, 150 or 200 µg/mL). Long-term amitriptyline treatment at doses 200 µg/mL alleviated the cuff-induced allodynia after 10 days of treatment (n = 5 mice/group, surgery x time interaction, $F_{9,216} = 2.4$, $p < 0.001$; post-hoc: ""CuffDul200" at $p < 0.001$ on post-surgery days 24 to 35) (**fig. S2A**). A partial antiallodynic effect was also present with the 150 and 100 µg/mL doses after 14 and 20 days of treatment respectively (post-hoc: "CuffSacch" < "CuffDul150" at $p < 0.05$ on post-surgery days 29 to 35 and "CuffSacch" < "CuffDul100" at $p < 0.005$ on post-surgery day 35). The 50 µg/mL dose of amitriptyline had no significant effect (**fig. S2A**). Treatments at different doses did not affect the contralateral nociceptive thresholds (**fig. S2A**). Chronic oral treatment with amitriptyline at 200 µg/mL did not affect mechanical thresholds of mice of the Sham group (**fig. S2A**).

Noradrenaline: peripheral vs. central mechanisms.

Since preclinical and clinical data suggest that the noradrenergic system is essential for the pain-relieving action of antidepressants, we investigated the possible source of endogenous noradrenaline which is used by acute and chronic duloxetine treatment to alleviate allodynia. For this purpose, peripheral noradrenergic lesions were done with guanethidine, a neurotoxin which does not cross the brain blood barrier and spinal noradrenergic lesions were done with 6-OHDA, two weeks before the induction of neuropathy. Neither peripheral nor spinal noradrenergic denervations modified the paw withdrawal thresholds in sham mice (**Fig. 1 A and B**). In nerve injured mice, peripheral depletion of noradrenaline abolished the long-term effect of duloxetine (n = 5-8 mice/group, surgery x time interaction, $F_{9,162} = 6.7$, $p < 0.001$; post-hoc: "CuffSacch" = "CuffDul200" at $p > 0.05$ on post-surgery days 8 to 42) (**Fig. 1A**), while it had no effect on the acute antiallodynic action of 30 mg/kg of duloxetine (n = 5-8 mice/group, surgery x time interaction, $F_{2,34} = 6.2$, $p < 0.001$; post-hoc: "CuffSacch" <



D

Noradrenaline (pmol/mg protein)

	Mean	SEM
Sham, no lesion	24.94	1.03
Cuff, no lesion	24.89	0.78
Cuff, peripheral lesion	24.19	2.46
Cuff, central lesion	7.49 ***	0.30

Serotonin (pmol/mg protein)

	Mean	SEM
Sham, no lesion	80.64	10.15
Cuff, no lesion	105.35 *	10.32
Cuff, peripheral lesion	65.12 **	25.44
Cuff, central lesion	58.46 *	3.72

Dopamine (pmol/mg protein)

	Mean	SEM
Sham, no lesion	43.06	2.17
Cuff, no lesion	44.43	4.68
Cuff, peripheral lesion	49.05	10.19
Cuff, central lesion	42.38	2.12

Fig. 1. Noradrenergic substrate for the antiallodynic action of duloxetine.
Guanethidine (peripheral lesion, 30 mg/kg, i.p.) and 6-OHDA (central lesion, 20 µg per mouse, i.t.) allowed lesioning the noradrenergic fibers. B corresponds to the mechanical threshold sensitivity before surgery. (**A**) Chronic duloxetine (200 µg/mL) exerts a delayed antiallodynic effect (top graph). The peripheral noradrenergic lesion (middle graph), but not the central one (bottom graph), suppresses this long-term antiallodynic action (chronic/acute n are given in bracket). (**B**) Acutely, a high dose of duloxetine (30 mg/kg) induces a rapid antiallodynic effect (top graph). This effect is affected by a central noradrenergic lesion (bottom graph), but not by a peripheral lesion (middle graph) (chronic/acute n are given in bracket). (**C**) Tyrosine hydroxylase (TH) immunostaining in the lumbar dorsal root ganglia (DRG; scale bars: 50 µm) and the lumbar spinal cord (SC; scale bars: 100 µm). The peripheral neuropathy induces a sympathetic sprouting in the DRG (white arrows). Peripheral noradrenergic lesion suppressed these fibers without affecting the spinal cord ones. Central lesion suppressed descending noradrenergic fibers (green arrows), sparing DRG sprouting. (**D**) Monoamines levels in the lumbar spinal cord were determinate. Intrathecal 6-OHDA significatively reduced the spinal noradrenaline levels. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to Sham Saccharin Groups.

"CuffDul30" at $p < 0.001$ on post-administration time 120 min) (**Fig. 1B**). When we suppressed the central noradrenergic fibers, chronic duloxetine was still effective (n = 5-7 mice/group, surgery x time interaction, $F_{9,153} = 8.8$, $p < 0.001$; post-hoc: "CuffSacch" < "CuffDul200" at $p < 0.005$ on post-surgery day 30 and at $p < 0.001$ on post-surgery days 34 to 41) (**Fig. 1A**), while the acute effect disappeared (n = 5-7 mice/group, surgery x time interaction, $F_{2,24} = 13.8$, $p < 0.001$; post-hoc: "CuffSacch" = "CuffDul30" at $p > 0.05$ on post-administration time 120 min) (**Fig. 1B**).

Similar results were observed with the tricyclic antidepressant amitriptyline. The peripheral injection of guanethidine prevented the antiallodynic action of long term oral treatment with amitriptyline (n = 5-8 mice/group, surgery x time interaction, $F_{9,162} = 6.8$, $p < 0.001$; post-hoc: "Cuff Sacch" = "Cuff Ami 200 µg/mL" at $p > 0.05$ on post-surgery days 0 to 41) (**fig. S2B**). On the contrary, an intrathecal injection at thoracic level abolished the acute antiallodynic effect induced by intraperitoneal administration of 15 mg/kg on paw withdrawal thresholds (n = 5-8 mice/group, surgery x time interaction, $F_{9,153} = 7.6$, $p < 0.001$; post-hoc: "Cuff Sacch" = "Cuff Ami" at $p > 0.05$ on post-administration time 60 min) (**fig. S2B**).

These lesions were neuroanatomically confirmed by TH immunohistochemistry (**Fig. 1C**). As previously reported (Ramer and Bisby, 1998; Bohren et al., 2013 *cf. p.16*) a noradrenergic sprouting was observed in nerve-injured mice. This noradrenergic sprouting in lumbar dorsal root ganglia remains present after thoracic intrathecal 6-OHDA lesion. In the spinal cord, the lumbar TH staining was not affected by the peripheral sympathectomy. However, a significant reduction of TH fibers was observed at lumbar spinal level following thoracic intrathecal lesions. 6-OHDA depleted the content of noradrenaline by more than 70% (n = 5-14 for each group, $F_{3,29} = 76.6$, $p < 0.001$; post-hoc: "Cuff, thoracic lesion" < "Cuff, no lesion" at $p < 0.001$) (**Fig. 1D**), with a significant reduction of serotonin (n = 5-14 for each group, $F_{3,29} = 13.4$, $p < 0.001$; post-hoc: "Cuff, thoracic lesion" < "Cuff, no lesion" at $p < 0.001$) and no effect on dopamine. Guanethidine had no effect on noradrenergic and dopaminergic spinal levels but led to a slight reduction on the content of spinal serotonin (n = 5-14 for each group, $F_{3,29} = 13.4$, $p < 0.001$; post-hoc: "Cuff, peripheral lesion" < "Cuff, no lesion" at $p < 0.001$).

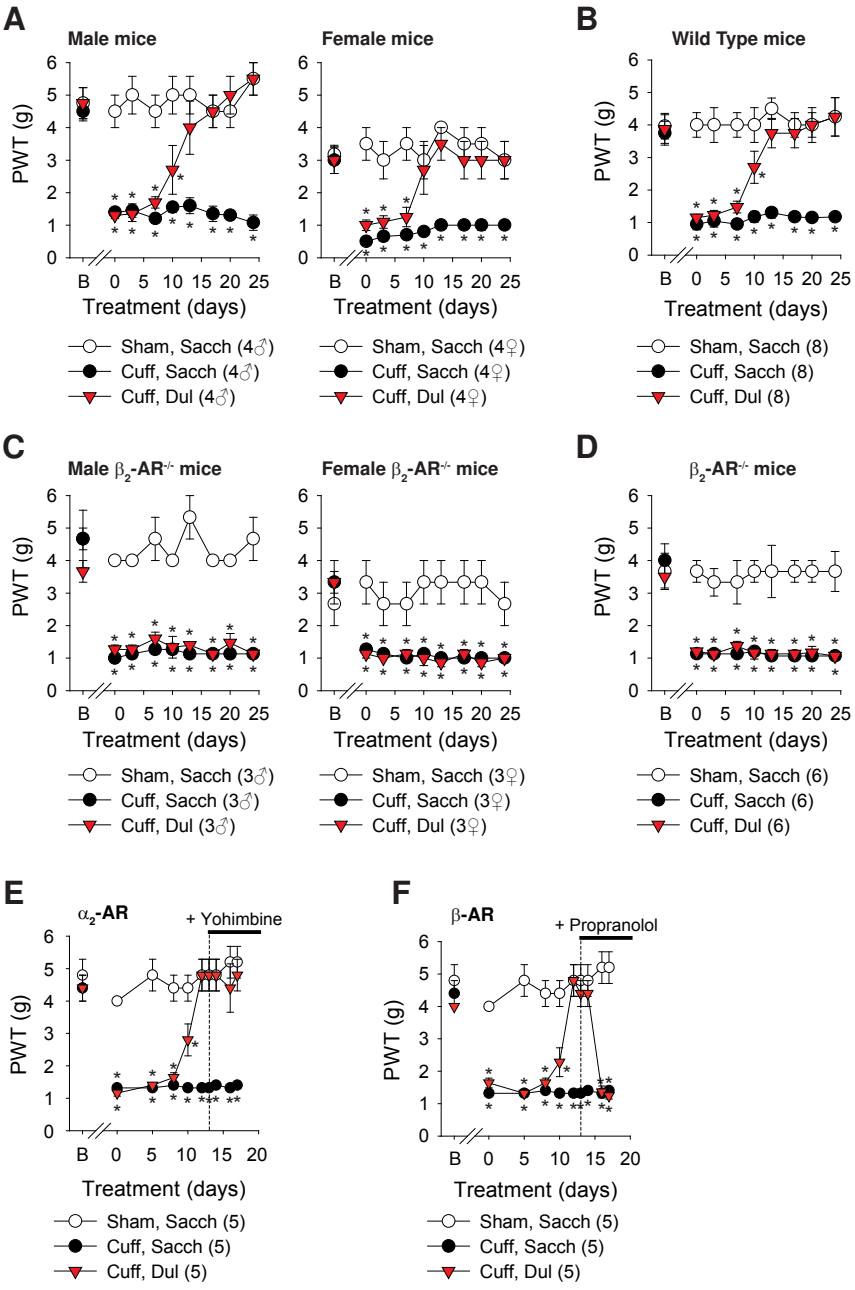


fig.S3. Role of α_2 and β_2-AR in antiallodynic effects of chronic oral duloxetine treatment.
(**A**) The mechanical sensitivity threshold of female mice is lower than those of male mice (male vs. female: $t_{90} = 8.5$, $P < 0.001$). However, both sexes developed mechanical allodynia and duloxetine (200 µg/mL) was effective in reversing the Cuff-induced allodynia in both wild-type male and female mice (n = 4/group, Male mice: surgery x time interaction, $F_{8,72} = 6.9$, $p < 0.001$; post-hoc: "Cuff Sacch" < "Cuff Dul 200 µg/mL" at $p < 0.001$ on treatment days 13 to 24; Female mice: surgery x time interaction, $F_{8,72} = 2.6$, $p < 0.005$; post-hoc: "Cuff Sacch" < "Cuff Dul 200 µg/mL" at $p < 0.005$ on treatment days 10 to 24). (**B**) Males and females were then pooled in each experimental group. Chronic duloxetine treatment abolishes the allodynia in wild-type mice. (**C**) Duloxetine was ineffective in reversing the Cuff-induced allodynia in both male and female mice (n = 3/group, Male mice: surgery x time interaction, $F_{8,48} = 3.6$, $p < 0.001$; post-hoc: "Sham Sacch" > "Cuff Dul 200 µg/mL" at $p < 0.001$ on treatment days 0 to 24; Female mice: surgery x time interaction, $F_{8,48} = 2.1$, $p < 0.05$; post-hoc: "Sham Sacch" > "Cuff Dul 200 µg/mL" at $p < 0.05$ on treatment days 0 to 24). (**D**) Chronic duloxetine treatment did not abolish the allodynia in β2-AR deficient mice. (Data are pooled from independents experiments, n are given in bracket; each final group includes the same number of male and female mice). (**E**) Chronic duloxetine relieves allodynia and yohimbine co-treatment has no effect. (**F**) Chronic duloxetine exerts an antiallodynic action and propranolol co-treatment reverses this effect. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to Sham Saccharin Groups. B corresponds to the mechanical threshold sensitivity before surgery.

Adrenoceptors: β_2-AR vs. α_{2A}-AR.

After long-term treatment, duloxetine reversed the cuff-induced allodynia in WT mice (n = 8 mice/group, surgery x time interaction, $F_{8,168}$ = 7.7, p < 0.001; post-hoc: "CuffSacch" < "CuffDul200" at p < 0.01 on treatment days 10 to 24), while the treatment remained ineffective in β_2-AR$^{-/-}$ mice, even after 25 days (n = 6 mice/group, surgery x time interaction, $F_{8,120}$ = 3.6, p < 0.001; post-hoc: "CuffDul200" < "ShamSacch" at p < 0.001 on treatment days 0 to 24) (**Fig. 2A**). Similar results were also observed with amitriptyline (n = 6-8 mice/group; WT: surgery x time interaction, $F_{8,168}$ = 9.2, p < 0.001; post-hoc: "CuffSacch" < "Ami200" at p < 0.005 on treatment days 13 to 24; β_2-AR$^{-/-}$: surgery x time interaction, $F_{8,120}$ = 3.6, p < 0.001; post-hoc: "CuffAmi200" < "ShamSacch" at p < 0.001 on treatment days 0 to 24) (**fig. S2C**). It also be noted that these results were obtained in male and female mice, being similar in both sexes (**fig. S3A, B, C and D**).

While β_2-AR appear to be essential to the chronic antiallodynic action of antidepressants, it is not the case for their acute effect. Indeed, acute administration of duloxetine at day 15 postsurgery transiently relieved mechanical allodynia 120 min after *per os* administration in both WT and β_2-AR$^{-/-}$ mice (n = 6-8 mice/group; WT: surgery x time interaction, $F_{2,42}$ = 12.6, p < 0.001; post-hoc: "CuffSacch" < "CuffDul30" at p < 0.001 on post-administration time 120 min; β_2-AR$^{-/-}$: surgery x time interaction, $F_{2,30}$ = 6.1, p < 0.005; post-hoc: "CuffSacch" < "CuffDul30" at p < 0.005 on post-administration time 120 min) (**Fig. 2B**). The chronic and the acute treatment with duloxetine had no influence on the nociceptive thresholds of Sham mice (**Fig. 2A and B**).

To further control whether the impact of the β_2-AR deletion not a developmental, we also tested a pharmacological antagonist. After chronic duloxetine treatment, a prolonged co-administration of the β-AR antagonist propranolol suppressed the antiallodynic effect of duloxetine (n = 5-8 mice/group, surgery x time interaction, $F_{1,16}$ = 10.6, p < 0.001; post-hoc: "CuffChronicDul before propranolol" > "CuffDul after propranolol" at p < 0.001 on day 6 after co-administration of propranolol) (**Fig. 2C**) but remained ineffective on its acute antiallodynic effect (**Fig. 2D**). The time course showed that this relapse of long term mechanical allodynia appeared after 4 days of propranolol co-treatment (n = 5 mice for each group, surgery x time interaction, $F_{9,108}$ = 6.4, p < 0.001; post-hoc: "CuffDul" = "CuffSacch" < "ShamSacch" at p < 0.001 on day 16 and 18), whereas no effect of yohimbine was found (**fig. S3F and E**).

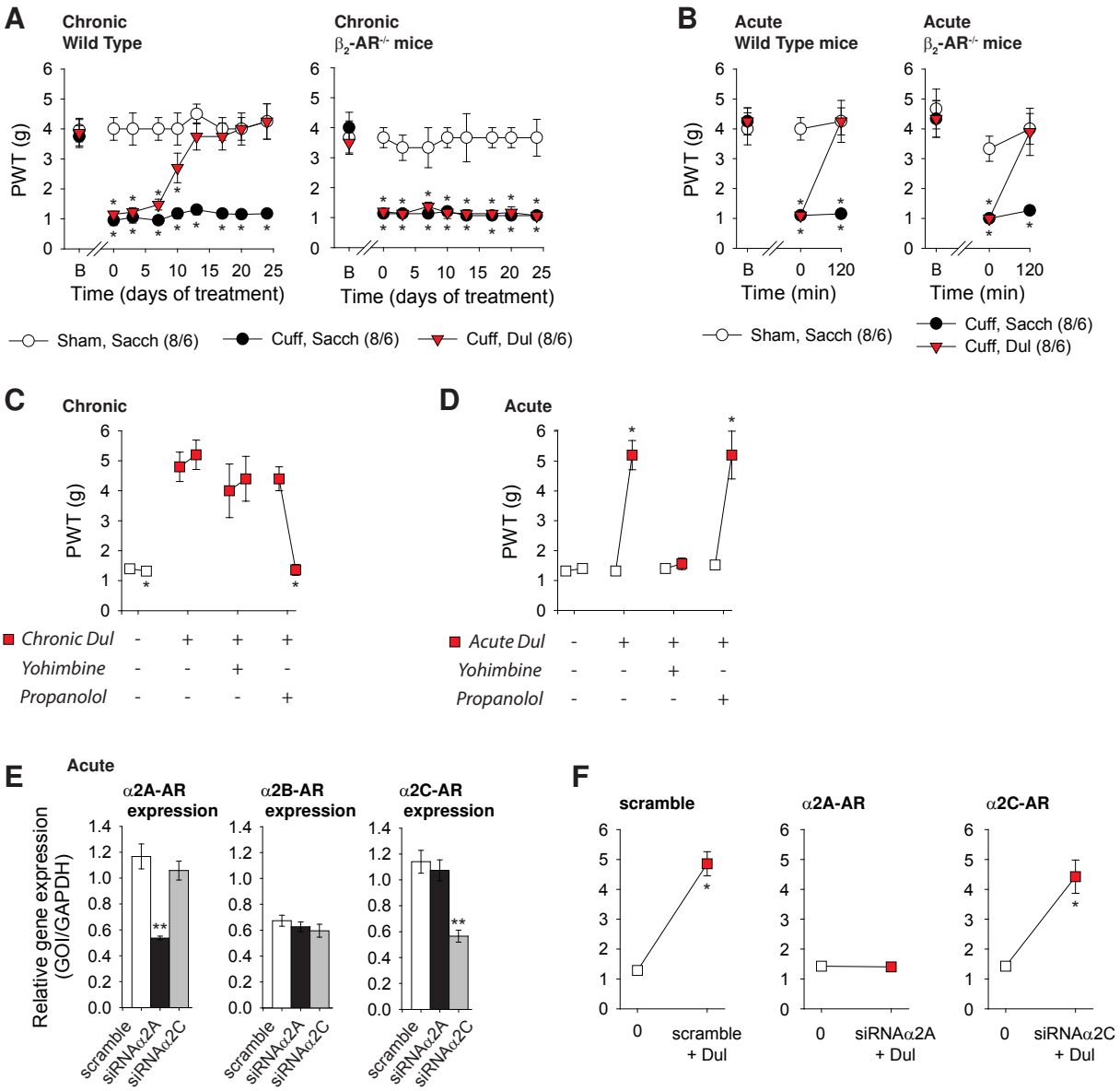


Fig. 2. Role of the α and β adrenoceptors on the antiallodynic effect of duloxetine.
(**A**) In wild type mice, chronic oral duloxetine treatment (200 µg/mL) suppresses the ipsilateral allodynia but remained ineffective in β2-AR deficient mice (WT/β2-AR$^{-/-}$ n are given in bracket), *p < 0.05 compared to Sham Saccharin Groups. (**B**) Acutely, a high dose of duloxetine (30 mg/kg) suppresses the mechanical allodyna in both wild-type and β2-AR deficient mice (WT/β2-AR$^{-/-}$ n are given in bracket), *p < 0.05 compared to Sham Saccharin Groups. (**C**) In nerve injured mice, propranolol (β-AR antagonist, 50 µg/mL) but not yohimbine (α2-AR antagonist, 20 µg/mL) suppresses the antiallodynic action of chronic duloxetine (n = 5-8 per groups), *p < 0.05 compared to time before antagonist administration. (**D**) In nerve injured mice, yohimbine but not propanolol suppresses the acute action of a high dose of duloxetine (n = 5-8 per groups), *p < 0.05 compared to time before acute administration. (**E**) Selectivity of α2A-AR and α2C-AR siRNA delivered intrathecally (1 nmol), **p < 0.01 compared to scramble Groups. (**F**) α2A-AR downexpression but not α2C-AR suppresses the acute effect of duloxetine (n = 5-8 per groups), *p < 0.05 compared to pre-administration treatments. Data are expressed as mean ± SEM. B corresponds to the mechanical threshold sensitivity before surgery.

Contrary to β_2-AR, α_2-AR appear to be involved in the acute but not chronic effect of duloxetine. Indeed, a prolonged co-administration of the α_2-AR antagonist, yohimbine, did not affect the action of long-term duloxetine (**Fig. 2C and fig.S3E**) but suppressed the acute antiallodynic effect of duloxetine (n = 5-8 mice/group, surgery x time interaction, $F_{1,16} = 15.3$, $p < 0.001$; post-hoc: "CuffAcuteDul alone" > "CuffAcuteDul after duloxetine/yohimbine" at $p < 0.001$ on post-administration time 120 min) (**Fig. 2D**).

To further explore the role of α_2-AR in acute duloxetine action, we used siRNA-mediated depletion to test the respective role of α_{2A}-AR and α_{2C}-AR in the spinal cord. Selective decrease in mRNA expression was present in nerve injured mice, seventy-two hours following siRNA intrathecal delivery (n = 6-8 mice/group; *adra2a*: $p < 0.001$; post-hoc: "siRNA α_{2A}" < "scramble = siRNA α_{2C}" at $p < 0.005$; $p < 0.001$; *adra2c*: post-hoc: "siRNA α_{2C}" < "scramble = siRNA α_{2A}" at $p < 0.01$) (**Fig. 2E**). The α_{2A}-AR, but not the α_{2C}-AR downregulation suppressed the acute antiallodynic effect of duloxetine (n = 5-8 mice/group, siRNA α_{2A}: surgery x time interaction, $F_{1,28} = 20.1$, $p < 0.001$; post-hoc: "Cuff siRNA α_{2A} + Dul" = "Cuff T0" at $p > 0.05$; siRNA α_{2C}: surgery x time interaction, $F_{1,28} = 20.1$, $p < 0.001$; post-hoc: "Cuff siRNA α_{2C} + Dul" > "Cuff T0" and "Cuff scramble + Dul" > "Cuff T0" at $p < 0.001$) (**Fig. 2F**).

Opioids receptors: MOP, DOP and KOP.

Most research groups who studied on the opioid system in antidepressants action on pain observed an implication of opioids receptors, but with discrepancies concerning the identity location of involved receptors. We have verified this involvement using transgenic mice and pharmacological tools.

Long-term duloxetine reversed allodynia in WT, $MOP^{-/-}$ and $KOP^{-/-}$ mice (n = 6-8 mice/group; WT: surgery x time interaction, $F_{8,168} = 6.6$, $p < 0.001$; post-hoc: "CuffSacch" < "CuffDul200" at $p < 0.005$ on treatment days 10 to 24; $MOP^{-/-}$: surgery x time interaction, $F_{8,120} = 5.4$, $p < 0.001$; post-hoc: "CuffSacch" < "CuffDul200" at $p < 0.005$ on treatment days 13 to 24; $KOP^{-/-}$: surgery x time interaction, $F_{8,120} = 3.1$, $p < 0.001$; post-hoc: "CuffSacch" < "CuffDul200" at $p < 0.05$ on treatment days 13 to 24), while the chronic treatment remained ineffective in $DOP^{-/-}$ mice (n = 6 mice/group, surgery x time interaction, $F_{8,120} = 3.8$, $p < 0.001$; post-hoc: "CuffDul200" < "ShamSacch" at $p < 0.001$ on treatment days 0 to 24) (**Fig. 3A**). Similar results were observed with amitriptyline (n = 6-8 mice/group; WT: surgery x time interaction, $F_{8,168} = 8.1$, $p < 0.001$; post-hoc: "CuffSacch" < "Ami200" at $p < 0.005$ on

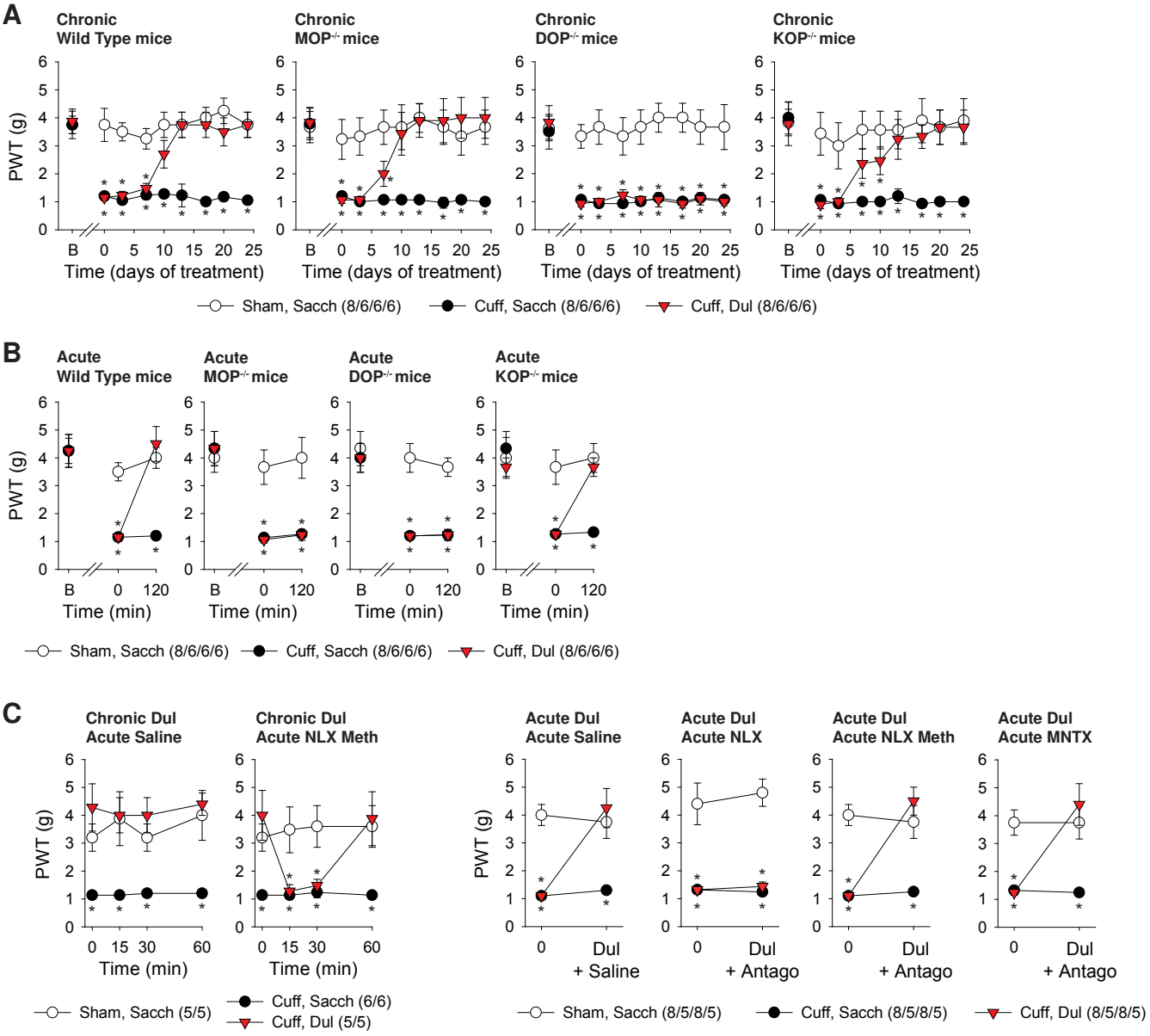
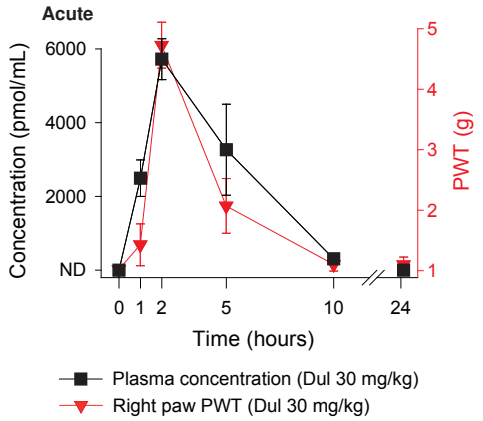


Fig. 3. Involvement of the opioid system in the antiallodynic effect of duloxetine.
(**A**) Chronic oral duloxetine (200 µg/mL) suppresses the ipsilateral allodynia in wild-type, in MOP-deficient and in KOP-deficient mice, but it remains ineffective in DOP-deficient mice (WT/MOP$^{-/-}$/DOP$^{-/-}$/KOP$^{-/-}$ n are given in bracket). (**B**) Acutely, a high dose of duloxetine (30 mg/kg) relieves mechanical allodynia in wild-type and in KOP-deficient mice, but not in DOP- and MOP-deficient mice (WT/MOP$^{-/-}$/DOP$^{-/-}$/KOP$^{-/-}$ n are given in bracket). (**C**) An acute injection of the peripheral opioid receptors antagonist, naloxone methiodide (NLX Meth, 5 mg/kg, s.c.) induces a relapse of the allodynia in chronically but not in acutely treated mice (Saline/NLX Meth n are given in bracket). An acute injection of peripheral opioid receptors antagonist, methylnaltrexone (MNTX, 10 mg/kg, s.c.) does not modify the acute effect of duloxetine but an acute injection of non selective opioid receptors antagonist, naloxone (NLX, 1 mg/kg, s.c.) suppressed this acute effect (Saline/NLX/NLX Meth/MNTX n are given in bracket). Data are expressed as mean ± SEM. *p < 0.05 compared to Sham Saccharin Groups. B corresponds to the mechanical threshold sensitivity before surgery.

treatment days 13 to 24; $MOP^{-/-}$: surgery x time interaction, $F_{8,120} = 4.5$, $p < 0.001$; post-hoc: "CuffSacch" < "Ami200" at $p < 0.05$ on treatment days 10 to 24; $KOP^{-/-}$: surgery x time interaction, $F_{8,120} = 8.2$, $p < 0.001$; post-hoc: "CuffSacch" < "Ami200" at $p < 0.05$ on treatment days 10 to 24; $DOP^{-/-}$: surgery x time interaction, $F_{8,120} = 3.4$, $p < 0.001$; post-hoc: "CuffAmi200" < "ShamSacch" at $p < 0.001$ on treatment days 0 to 24) (**fig. S2E**). Acute duloxetine at day 15 postsurgery transiently relieved mechanical allodynia 120 min after *per os* administration in both WT and $KOP^{-/-}$ mice (n = 6-8 mice/group; WT: surgery x time interaction, $F_{2,42} = 10.7$, $p < 0.001$; post-hoc: "CuffSacch" < "CuffDul30" at $p < 0.001$ on post-administration time 120 min; $KOP^{-/-}$: surgery x time interaction, $F_{2,30} = 8.7$, $p < 0.001$; post-hoc: "CuffSacch" < "CuffDul30" at $p < 0.005$ on post-administration time 120 min). Moreover, the acute treatment remained ineffective in $MOP^{-/-}$ and $DOP^{-/-}$ mice (n = 6-8 mice/group; $MOP^{-/-}$: surgery x time interaction, $F_{2,30} = 5.6$, $p < 0.005$; post-hoc: "CuffDul30" < "ShamSacch" at $p < 0.001$ on post-administration time 0 to 120; $DOP^{-/-}$: surgery x time interaction, $F_{2,30} = 5.0$, $p < 0.005$; post-hoc: "CuffDul30" < "ShamSacch" at $p < 0.001$ on post-administration time 0 to 120) (**Fig. 3B**).

We then tested whether the implication of opioid receptors was peripheral or central. The acute injection of a peripheral antagonist, naloxone methiodide, induced a relapse of allodynia in nerve injured mice chronically treated with duloxetine (ChronicDul Acute NLX Meth: n = 5 mice/group, surgery x time interaction, $F_{3,39} = 7.1$, $p < 0.001$; post-hoc: "CuffDul200" < "ShamSacch" at $p < 0.05$ on post NLX Meth administration time 15 to 30) (**Fig. 3C**). Similar results were observed with long-term amitriptyline (n = 5-6 mice/group, surgery x time interaction, $F_{3,39} = 8.8$, $p < 0.001$; post-hoc: "CuffAmi200" < "ShamSacch" at $p < 0.005$ on post NLX Meth administration time 15 to 30) (**fig. S2D**). While naloxone, a non-selective antagonist of opioid receptors, suppressed the acute effect of duloxetine; neither naloxone, nor the peripheral opioid receptors antagonist methylnaltrexone, did affect this acute action (n = 5-8 mice/group; Acute Dul Acute NLX: surgery x time interaction, $F_{1,15} = 19.5$, $p < 0.001$; post-hoc: "CuffDul30" < "ShamSacch" at $p < 0.001$ on post Dul + NLX administration; Acute Dul Acute NLX Meth: surgery x time interaction, $F_{1,21} = 19.0$, $p < 0.001$; post-hoc: "CuffDul30" > "CuffSacch" at $p < 0.001$ on post Dul + NLX Meth administration; Acute Dul Acute MNTX: surgery x time interaction, $F_{1,15} = 19.3$, $p < 0.001$; post-hoc: "CuffDul30" > "CuffSacch" at $p < 0.001$ on post Dul + MNTX administration) (**Fig. 3C**).

A



B

Groups	Mean (pmol/mL)	SEM
Mice (Cuff)		
Acute (n=7-8)		
T1h	2492.06 (2.5 µM)	494.89
T2h	5721.58 (5.8 µM)	554.14
T5h	3263.69 (3.3 µM)	1233.56
T10h	305.62 (0.3 µM)	26.69
T24h	ND	ND
Chronic (n=5)		
Night phase	453.20 (0.5 µM)	46.44
Day phase	376.05 (0.4 µM)	65.09
Human (n=4)		
Chronic, acute peak (6h)	173.81 (0.2 µM)	10.97
Chronic, residual	32.49 (0.03 µM)	2.09
Plasma elimination half-life	5.8 h	0.09

Fig. 4. Duloxetine plasma concentrations in mice and in human patients.
(**A**) Mean plasma concentration-time profile and behavioral data in mice for acute duloxetine oral administration (30 mg/kg). (**B**) Mean plasma concentration of duloxetine in mice, 1, 2, 5, 10 and 24 hours after acute oral administration (30 mg/kg); in mice after a chronic oral treatment (200 µg/mL); the plasma of these animals was collected 3 hours after lights out (night phase) or 6 hours after lights on (day phase); in neuropathic pain patients 6 hours after one of the daily administration of the chronic treatment (60 mg per day) or the morning just before taking treatment (n are given in bracket). Data are expressed as mean ± SEM. Abbreviation: ND, undetectable.

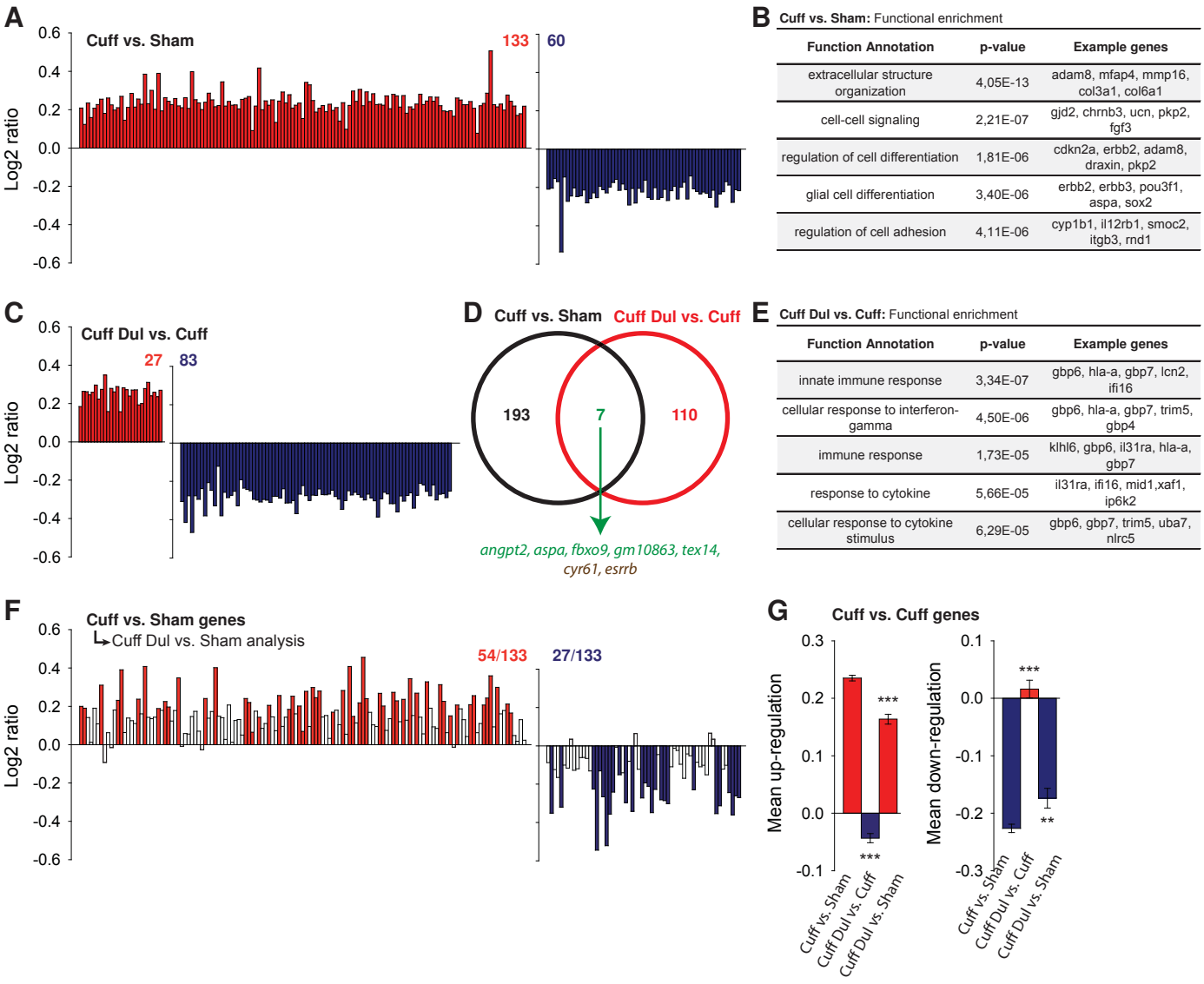
These data show that the long-term action of duloxetine depends on the presence of peripheral DOP receptors, while acute effect at high dose requires central MOP and DOP receptors.

Plasma concentrations of duloxetine in patients and in mice.

Together the previous findings highlighted two independents mechanisms, both starting with the recruitment of noradrenaline, in the action of duloxetine: 1) A delayed antiallodynic action, which is peripherally mediated and involves β_2-AR and DOP receptors; 2) An acute transitory antiallodynic effect that depends on the central noradrenergic descending pathways, is α_{2A}-AR mediated and requires the presence of central MOP and DOP receptors. These results raise the question of whether one or both mechanisms are present in neuropathic pain patients chronically treated by duloxetine. To address the clinical relevance of the animal procedure, we used mass spectrometry to compare plasma levels of duloxetine.

In mice with nerve injury, there is a direct correlation between plasmatic levels of acutely-delivered duloxetine and the paw withdrawal thresholds ($r > 0.8$) (**Fig. 4A**). A full recovery for neuropathic allodynia was observed with plasmatic concentrations of 5721 ± 554 pmol/mL, corresponding to 2 hours after administration (n = 7-8 mice/group, surgery x time interaction, $F_{1,55} = 39.6$, $p < 0.001$; post-hoc: "CuffDul30 T0h" and "CuffDul30 T1h" and "CuffDul30 T10h" and "CuffDul30 T24h" < "CuffDul30 T2h" at $p < 0.005$). However, plasma levels of nerve injured mice receiving prolonged treatment with duloxetine and displaying an antiallodynic effect were between 376 ± 65 (sample taken during the rest phase of the animal = day) and 453 ± 46 pmol/mL (sample taken during the active phase of the animal = night), which is 12 times lower than observed acutely (**Fig. 4B**).

In patients, the peak plasma concentration of duloxetine is present 6 hours after taking the treatment (Senthamil Selvan et al., 2007). We evaluated the residual plasmatic concentration (ie before the morning dose of duloxetine) and the plasmatic peak concentration (ie 6 hours after the morning dose of duloxetine) in patients relieved of at least 30% of their neuropathic pain with chronic duloxetine treatment. Four patients, 28-66 years old, were included in this pharmacokinetic evaluation of duloxetine (**Table S1**). In humans, after chronic duloxetine treatment (60 mg, a morning taking), the plasmatic peak concentration was 173.81 ± 10.97 pmol/mL and the residual plasmatic concentration was



Function Annotation	p-value	Example genes
extracellular structure organization	4,05E-13	adam8, mfap4, mmp16, col3a1, col6a1
cell-cell signaling	2,21E-07	gjd2, chrnb3, ucn, pkp2, fgf3
regulation of cell differentiation	1,81E-06	cdkn2a, erbb2, adam8, draxin, pkp2
glial cell differentiation	3,40E-06	erbb2, erbb3, pou3f1, aspa, sox2
regulation of cell adhesion	4,11E-06	cyp1b1, il12rb1, smoc2, itgb3, rnd1

Cuff Dul vs. Cuff: Functional enrichment

Function Annotation	p-value	Example genes
innate immune response	3,34E-07	gbp6, hla-a, gbp7, lcn2, ifi16
cellular response to interferon-gamma	4,50E-06	gbp6, hla-a, gbp7, trim5, gbp4
immune response	1,73E-05	klhl6, gbp6, il31ra, hla-a, gbp7
response to cytokine	5,66E-05	il31ra, ifi16, mid1,xaf1, ip6k2
cellular response to cytokine stimulus	6,29E-05	gbp6, gbp7, trim5, uba7, nlrc5

Fig. 5. Transcriptomic analysis of nerve injury and duloxetine impact on dorsal root ganglia.
(**A**) Genes significantly upregulated (red) and downregulated (blue) in dorsal root ganglia following nerve injury. n represents the number of significant genes. (**B**) The table shows the top enriched functional terms in dorsal root ganglia following nerve injury. (**C**) Genes significantly upregulated and downregulated in dorsal root ganglia following long-term duloxetine treatment. n represents the number of significant genes. (**D**) The diagram shows overlap containing significant genes. In green are represented genes which are corrected by treatment and in brown genes who are further underexpressed. (**E**) The table shows the top enriched functional terms in dorsal root ganglia following long-term duloxetine treatment. (**F**) Analysis of treatment effect in the initially cuff versus sham significant genes. (**G**) Average of up and down regulation in dorsal root ganglia following nerve injury in the initially cuff versus sham significant genes. Data are expressed as mean ± SEM.

32.49 ± 2.09 pmol/mL (**Fig. 4B**). In patients, plasma concentrations are below and compatible with the plasma concentrations achieved in animals under prolonged treatment.

Genome wide analysis of chronic duloxetine impact.

To go further in understanding this peripheral mechanism of prolonged treatment, we then conducted a transcriptomic analysis.

In dorsal root ganglia, following nerve injury, we observed significant changes in the expression of 193 genes, 2/3 of them being upregulated (**Fig. 5A, Cuff vs. Sham**). The functional analysis reveals that these modifications correspond to a remodeling of dorsal root ganglia, with a particular impact on the extracellular matrix, the integrine signaling and cell differentiation (**Fig. 5B**). The impact of duloxetine treatment however differs from that of the nerve injury. Indeed, twice fewer genes are significantly impacted by duloxetine, and 2/3 of them correspond to a downregulation of gene expression (**Fig. 5C**). Interestingly, only a small number of genes (7) significantly impacted by the nerve injury are also significantly regulated by the treatment (**Fig. 5D**). This corresponds to a corrective downregulation of *angpt2*, *aspa* and *gm10863*, to a corrective upregulation of *fbxo9* and *tex14*; and to an increased downregulation for *cyr61* and *esrrb*. In fact, the functional analysis reveals that duloxetine primarily affect neurogenic inflammation, as reflected by the presence of genes regulating inflammation via immune response or cytokine response (**Fig. 5E**).

While few of nerve injured-related genes were significantly altered by the treatment, chronic duloxetine mainly blunts the over and under-expression of a half of these nerve injured-related genes. As a consequence, only 20% of nerve injured-related genes still display significant changes following chronic duloxetine treatment (**Fig. 5F**). This leads duloxetine treatment to decrease the mean up- and down-regulation of genes affected by the sciatic nerve compression (**Fig. 5G**).

Role of TNFα and the NFκB pathway.

The impact of chronic duloxetine treatment on neurogenic inflammation is further supported by GSEA analyses. Indeed, significant enrichment of TNFα signaling via NF-κB is observed following nerve injury, while a downregulation is associated with chronic duloxetine treatment (**Fig. 6A**).

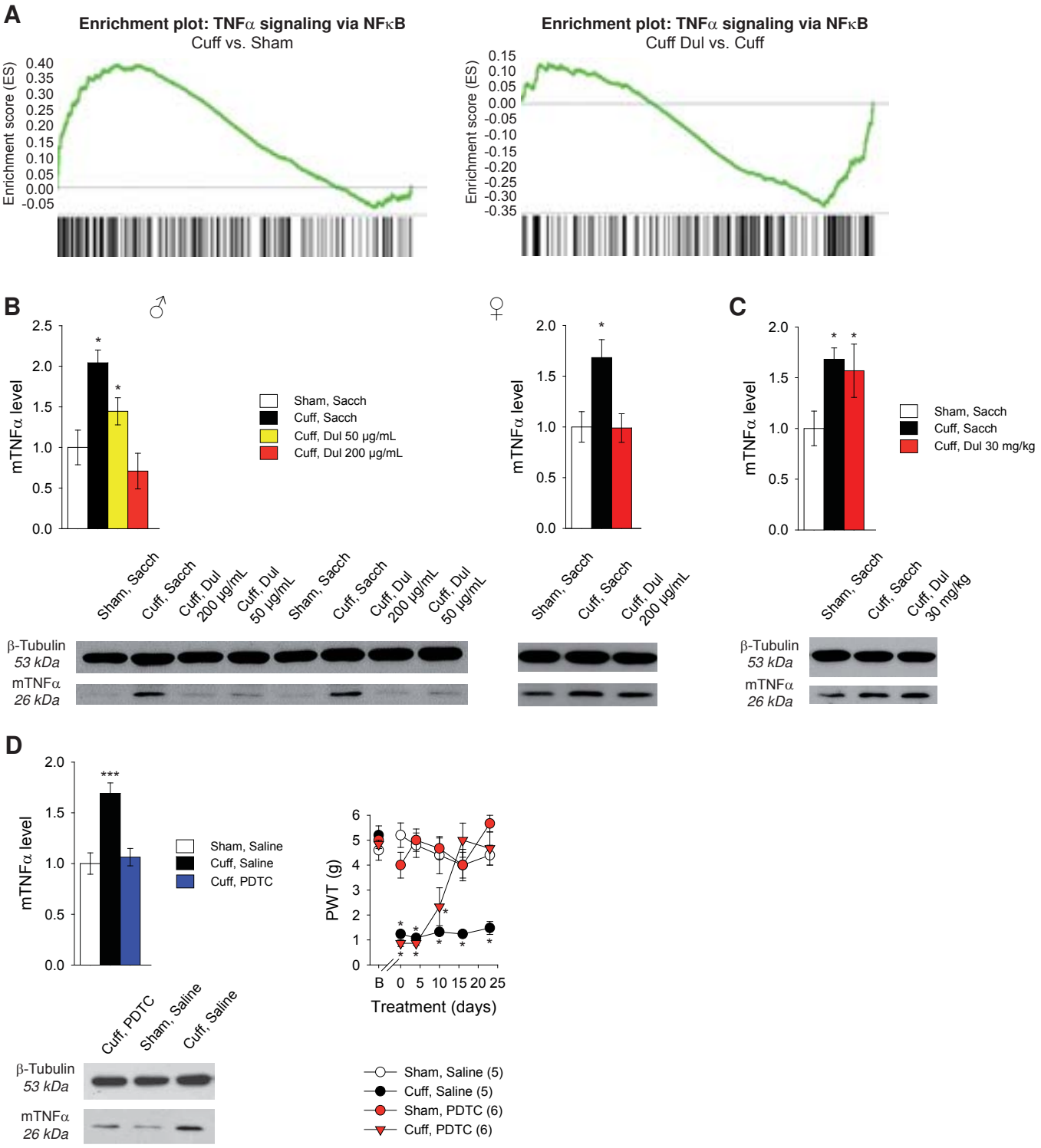


Fig. 6. Chronic duloxetine impact on the TNFα signaling via NFκB.
(**A**) At gene expression level, peripheral nerve injury induces an upregulation of the TNFα signaling via NFκB, while chronic duloxetine downregulates this signalization. (**B**) Western blot analysis on dorsal root ganglia showing an increase of TNFα levels in males and females mice after nerve injury, and an anti-TNFα action of the long-term duloxetine treatment (200 µg/mL) (n=7-9 per group). (**C**) Acute duloxetine treatment (30 mg/kg) does not impact the increased TNFα (n=7 per group). (**D**) Chronic pyrrolidine dithiocarbamate (PDTC) treatment suppressed the neuropathy-induced TNFα overexpression in the dorsal root ganglia and exerts an antiallodynic action (n are given in bracket). B corresponds to the mechanical threshold sensitivity before surgery. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

Using Western blot, an overexpression of the mTNFα is observed in the lumbar dorsal root ganglia of nerve injured male mice, which is dose-dependently corrected by chronic duloxetine treatment ($p < 0.001$; post-hoc: "CuffSacch" or "CuffDul50" > "CuffDul200" or "ShamSacch" at $p < 0.05$) (**Fig. 6B**). Similar results were found in female mice ($p < 0.05$; post-hoc: "CuffSacch" > "CuffDul200" or "ShamSacch" at $p < 0.05$) and with amitriptyline treatment ($p < 0.05$; post-hoc: "CuffSacch" > "CuffAmi200" or "ShamSacch" at $p < 0.05$) (**fig. S4**). Interestingly, 2 hours after acute administration of duloxetine, this anti-TNFα effect was not yet present at protein level ($p < 0.05$; post-hoc: "CuffSacch" or "CuffDul200" > "ShamSacch" at $p < 0.01$) (**Fig. 6C**).

As TNFα recruitment is accompanied by a recruitment of NFκB pathway, we tested the implication of this pathway with a chronic treatment by an inhibitor, the PDTC. We observed that PDTC suppressed both the nerve-injury induced increase in TNFα level ($p < 0.005$; post-hoc: "CuffSacch" > "CuffDul200" or "ShamSacch" at $p < 0.01$) and the mechanical hypersensitivity (n = 5-8 mice for each group, surgery x time interaction, $F_{5,90} = 7.7$, $p < 0.001$; post-hoc: "CuffSaline" < "CuffPDTC" at $p < 0.001$ on treatment days 16 to 23) (**Fig. 6D**).

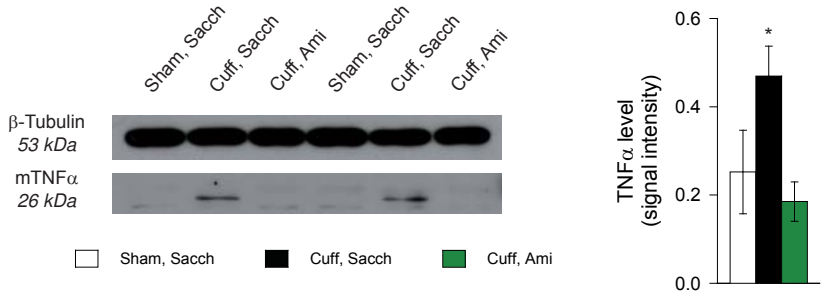


fig. S4. Impact of chronic amitriptyline treament on TNFα levels in dorsal root ganglia.
Western blot analysis on dorsal root ganglia showing increased TNFα levels in males mice with nerve injury and an anti-TNFα action of the long-term amitriptyline treatment (200 µg/mL). The histogram represents the relative amount of TNFα according to the density of the control group Sham Saccharin (n=5/group). Data are expressed as mean ± SEM.*p < 0.05 compared to the Sham Saccharin group.

Discussion

Les résultats de cette étude révèlent une dualité de mécanismes d'action de la duloxétine selon la dose administrée et la durée du traitement. Un traitement aigu à fortes doses ou prolongé à faibles doses ne fait pas intervenir les mêmes effecteurs. Nous avons mis en évidence l'existence d'un mécanisme d'action périphérique de la duloxétine, lors d'un traitement prolongé à faibles doses, impliquant la noradrénaline du système sympathique, les β_2-AR des ganglions rachidiens et les récepteurs DOP périphériques. A côté de ce mécanisme périphérique, il existe également un mécanisme central de la duloxétine, lorsque cette dernière est administrée à fortes doses, impliquant cette fois les contrôles descendants noradrénergiques, les α_{2A}-AR spinaux et les récepteurs MOP et DOP. En comparant les taux plasmatiques de duloxétine, il semble que le mécanisme périphérique soit plus proche de celui impliqué chez le patient. Pour aller plus loin dans la compréhension de ce mécanisme d'action présumé périphérique, nous avons effectué une analyse transcriptomique qui rapporte un effet anti-neuroinflammatoire périphérique, notamment par l'inhibition indirecte de la voie de signalisation TNFα-NFκB.

Effet analgésique et antiallodynique de la duloxétine.

Si l'indication première des antidépresseurs reste la dépression, certains d'entre eux ont une prescription plus étendue. Leur intérêt dans le traitement des douleurs neuropathiques est apparu dès 1960, suite à des observations rapportant un effet analgésique de l'imipramine chez des patients atteints de douleurs neuropathiques (Paoli et al., 1960). De nombreuses études cliniques ont été menées depuis (Watson et al., 1982; Sindrup et al., 2005; Saarto and Wiffen, 2007; Finnerup et al., 2015) et les antidépresseurs apparaissent efficaces dans un certain nombre de syndromes douloureux chroniques et ceci indépendamment de leurs effets thymoanaleptiques (Mico et al., 2006; Perahia et al., 2006). Dans notre modèle de douleur neuropathique périphérique, nous observons un soulagement durable de l'allodynie mécanique, dose-dépendant, induit par la duloxétine et l'amitriptyline. Une dose de 200 µg/mL suffit à cet effet thérapeutique, mais un délai de plusieurs jours est nécessaire, laissant supposer la mise en place de mécanismes de plasticité comme cela a pu être décrit pour la dépression (Vialou et al., 2013; Duman and Duman, 2015; Rantamaki and Yalcin, 2016). Nous avons ensuite mis en évidence l'effet analgésique,

ID Patient	Gender	Age (years)	Weight (kg)	Ethiology of NP	Location of NP	Treatment any time	NRS before duloxetine treatment	NRS after duloxetine treatment	peak plasma level	residual plasma level
1	M	66	76	PN Diabetic	Lower limbs	Baclofen 10 mg	8	1	166,95	36,25
2	F	56	72	PN Idiopathic	Lower limbs	Lyrica 150 mg	6	2	159,19	27,91
3	F	29	68	Radiculalgia Traumatic	L3	Lyrica 100 mg	6	1	195,30	30,00
4	F	43	55	Radiculalgia Traumatic	C7	None	8	4	NM	35,80

Table S1. Characteristics of patients included in the duloxetine plasma concentrations study.
Abbreviations: ID, identity; NM, not measured; NP, neuropathic pain; NRS, numeric rating scale; PN polyneuropathy.

là encore dose-dépendant, d'une administration unique de duloxétine ou d'amitriptyline. Dans ce cas, la dose nécessaire à l'action thérapeutique est plus élevée, 30 mg/kg pour la duloxétine, 15 mg/kg pour l'amitriptyline, et l'effet est transitoire, disparaissant dans les heures suivant l'administration.

Substrat noradrénergique impliqué.

La noradrénaline est un élément crucial dans l'action antiallodynique des antidépresseurs et l'hypothèse la plus commune dans la littérature avance que l'action analgésique de ces molécules impliquerait le recrutement des contrôles descendants provenant du locus cœruleus (Mico et al., 2006). Ainsi, il a été montré qu'une section des voies bulbospinales véhiculées par le faisceau dorsolatéral supprimait les effets thérapeutiques d'une administration aigüe de clomipramine (Ardid et al., 1995). Suite à une lésion nerveuse périphérique, un bourgeonnement des fibres descendantes au niveau spinal a également été observé et associé à une augmentation des taux extracellulaires de noradrénaline (Ma and Eisenach, 2003; Hayashida et al., 2008). Par des lésions noradrénergiques sélectives, ciblant les fibres descendantes noradrénergiques au niveau thoracique, nous mettons en évidence une implication de ces contrôles inhibiteurs dans l'analgésie aigüe, à fortes doses, de la duloxétine et de l'amitriptyline. Pour les mécanismes antinociceptifs avancés, des auteurs ont décrit un rôle des α_2-AR (Hajhashemi et al., 2014; Nakamura et al., 2014; Hughes et al., 2015). Ainsi, l'analgésie aigüe de l'amitriptyline est absente chez les souris α_{2A}-AR$^{-/-}$ non douloureuses (Ozdogan et al., 2004). Ceci est en accord avec nos résultats puisque dans notre modèle de douleur neuropathique périphérique la noradrénaline agit plus précisément via les α_{2A}-AR mais pas les α_{2c}-AR.

Bien que la plupart des études sur les antidépresseurs se soient concentrées sur leur action centrale, un effet périphérique a également été suggéré (Mico et al., 2006; Bohren et al., 2013). Nos résultats montrent que lors d'un traitement prolongé par la duloxétine à faibles doses et nécessitant un délai thérapeutique, la noradrénaline indirectement recrutée provient du système sympathique périphérique. En effet, suite à une lésion nerveuse périphérique, un bourgeonnement des fibres sympathiques se forme dans les ganglions rachidiens (McLachlan et al., 1993; Bohren et al., 2013). Le mécanisme antalgique fait ici intervenir les β_2-AR, qui se situent sur les cellules non neuronales des ganglions rachidiens (Bohren et al., 2013) et non les α_2-AR. Il est à noter que, dans ce cas, les taux sanguins se

rapprochent plus de ceux observés en clinique ; les antidépresseurs étant administrés de façon répétée, à doses moyennes, pendant plusieurs semaines. Nos résultats suggèrent donc le recrutement de deux substrats noradrénergiques différents, l'un central et l'autre périphérique, selon la durée et la dose du traitement.

Système opioïdergique et récepteurs impliqués.

Le système opioïdergique endogène via ses récepteurs MOP, DOP et KOP joue un rôle essentiel dans le contrôle de la douleur (Mogil et al., 2000; Gaveriaux-Ruff and Kieffer, 2002; Dierich and Kieffer, 2004). La plupart des études ont montré un recrutement de ce système (Valverde et al., 1994; Gray et al., 1998; Benbouzid et al., 2008a; Benbouzid et al., 2008b; Wattiez et al., 2011; Ucel et al., 2015), alors que quelques autres n'ont pas observé d'implication de ce système dans l'action antiallodynique et / ou antihyperalgique des antidépresseurs (Spiegel et al., 1983; Ghelardini et al., 2000). L'identité des récepteurs impliqués reste également un sujet discuté. Pour certains auteurs, utilisant des techniques pharmacologiques, le mécanisme d'action des antidépresseurs ferait intervenir les récepteurs DOP supraspinaux et MOP spinaux (Marchand et al., 2003) ; alors que pour d'autres, utilisant des animaux transgéniques, cette action dépendrait uniquement des récepteurs DOP (Benbouzid et al., 2008b; Bohren et al., 2010; Megat et al., 2015). Une étude propose même que l'implication du système opioïdergique soit différente selon le modèle de douleur neuropathique utilisé (Wattiez et al., 2011). Nos résultats sont en faveur d'un recrutement différent des récepteurs des opioïdes selon la dose administrée et la durée du traitement. Lors d'un traitement prolongé par la duloxétine ou l'amitriptyline, à faibles doses, le mécanisme antiallodynique fait intervenir les récepteurs DOP périphériques. Ces résultats sont conformes aux données de la littérature utilisant des animaux transgéniques et un traitement prolongé par la nortriptyline (Benbouzid et al., 2008b; Bohren et al., 2010; Megat et al., 2015). A fortes doses, le mécanisme d'action de la duloxétine implique à la fois les récepteurs MOP et DOP centraux. Ceci est en accord avec la littérature mettant en avant le rôle des récepteurs MOP spinaux et DOP supraspinaux lors d'un traitement sub-chronique par la clomipramine. En effet, un traitement sub-chronique de 24h se rapproche plutôt d'une administration aigüe par les taux sanguins qu'elle atteint et ne permet pas la mise en place des mécanismes de plasticité nécessaires à l'action à long terme des antidépresseurs. Nos résultats suggèrent donc un recrutement différent du système opioïdergique endogène,

central via les récepteurs MOP et DOP, et périphérique via les récepteurs DOP uniquement, selon la durée et la dose du traitement.

Concentrations plasmatiques de duloxétine chez le patient et chez la souris.

Les résultats obtenus en recherche préclinique sur les effets des antidépresseurs sur la douleur neuropathique sont parfois difficilement généralisables en clinique. Ces molécules sont prescrites sur de longues durées pour traiter des douleurs chroniques. Nos résultats, avec un traitement prolongé de duloxétine dans l'eau de boisson, reproduisent assez bien les données cliniques avec un délai thérapeutique semblable à celui décrit chez les patients. Toutefois nous avons décrit deux mécanismes d'action de la duloxétine, central et périphérique. Il est alors essentiel de se demander lequel de ces deux mécanismes est mis en jeu chez le patient soulagé de ses douleurs neuropathiques périphériques par la prise quotidienne d'une dose de 60 mg de duloxétine.

Il est difficile de comparer les doses administrées chez l'animal et chez l'homme notamment à cause de l'absorption intestinale du médicament, de son métabolisme et de sa biodisponibilité. Pour contourner ce problème, nous avons directement réalisé des dosages plasmatiques de la duloxétine chez les patients, au pic plasmatique et au taux résiduel, et nous les avons comparés à ceux obtenus chez l'animal sous traitement aigu ou prolongé. Nos résultats montrent que les concentrations plasmatiques de duloxétine chez le patient (entre 0,03 et 0,2 µM) sont proches, et même plus faibles, que celles observées chez l'animal sous traitement prolongé à faibles doses (entre 0,4 et 0,5 µM). Sous traitement aigu, à fortes doses, les concentrations plasmatiques mesurées chez l'animal sont par contre plus importantes (5,8 µM soit environ 12 fois plus élevées que lors d'un traitement prolongé), qu'en clinique et ne peuvent pas être atteintes chez un patient avec une posologie de 60 mg par jour. On peut donc penser que la probabilité d'un mécanisme d'action central de la duloxétine par recrutement des contrôles descendants noradrénergiques inhibiteurs chez le patient via une action sur les α_{2A}-AR est faible. Ce constat est également étayé par le fait que la duloxétine possède une demi-vie assez courte, entre 8 et 17 heures (en moyenne 12 heures) chez le sujet sain (Senthamil Selvan et al., 2007). Dans le cas d'un patient souffrant de douleurs neuropathiques, nous observons une réduction de cette demi-vie, aux alentours de 6 heures, sans doute par une accélération du métabolisme ; cela explique le fait que lorsque l'on réalise le prélèvement sanguin le matin, avant la prise du comprimé, les taux

plasmatiques résiduels soient si bas. Dans ces conditions, si les contrôles descendants étaient effectivement recrutés dans le mécanisme d'action de la duloxétine, on devrait s'attendre à un soulagement transitoire des patients, avec un effet antalgique fluctuant au cours du nycthémère et un effet maximal dans les 6 heures suivant la prise du comprimé. Ceci ne semble pas le cas, car lorsque nous interrogeons les patients de l'étude, leur intensité douloureuse reste relativement constante au cours de la journée. L'ensemble de nos résultats nous apporte des arguments, certes indirects, sur le mécanisme d'action de la duloxétine chez l'homme qui impliquerait plutôt la noradrénaline du système sympathique périphérique, les β_2-AR des ganglions rachidiens et les récepteurs DOP.

Nous pouvons par contre, pour différentes raisons, envisager que cela soit différent pour les antidépresseurs tricycliques, tels que l'amitriptyline. Les métabolites de l'amitriptyline sont des métabolites dits actifs, contrairement à ceux de la duloxétine qui sont considérés comme inactifs. Ainsi, l'amitriptyline, dont la demi-vie est comprise entre 22 et 40 heures, est transformée par le foie en nortriptyline, se liant également au transporteur de recapture de la noradrénaline et de la sérotonine. La demi-vie de ce métabolite est très longue (entre 16 et 90 heures), supérieure même à celle de la molécule mère, ce qui peut conduire à une demi-vie totale de plusieurs jours lorsque l'on ajoute l'effet de l'amitriptyline et de ses métabolites actifs. En outre, une titration des antidépresseurs tricycliques, avec augmentation des doses, est couramment réalisée et vivement conseillée en clinique, afin de permettre un soulagement efficace des patients tout en évitant de graves complications dues aux effets indésirables. Concernant la duloxétine, cette titration n'est pas nécessaire. Enfin, en clinique, le critère d'efficacité d'un traitement par antidépresseurs se traduit par une diminution supérieure à 30% des signes et des symptômes douloureux décrits par le patient. Dans notre modèle, nous ne mesurons qu'un seul paramètre, l'allodynie mécanique statique, mais on observe plusieurs cas de figures : 1) les animaux sont complètement soulagés de leur allodynie et retrouvent des valeurs de seuil de sensibilité mécanique comparables à celles obtenues chez les animaux contrôles ; 2) les animaux ne sont que partiellement soulagés avec des valeurs en dessous des animaux contrôles, mais toujours significativement supérieures aux valeurs des animaux neuropathiques non traités ; 3) les animaux présentent toujours une allodynie importante, comparable à celle des animaux neuropathiques non traités. Si l'on considère le critère d'efficacité clinique de 30%, les animaux se plaçant dans le second cas de figure peuvent être considérés comme soulagés,

avec des taux plasmatiques avoisinant les 5,8 µM, mais là encore on est nettement supérieur aux concentrations plasmatiques atteintes avec un traitement résiduel. Par contre, pour l'amitriptyline dont les métabolites sont actifs, on pourrait atteindre des taux avoisinants et une composante centrale pourrait donc être co-présente avec la composante périphérique. C'est probablement en raison de tous ces paramètres que l'amitriptyline, et en général les antidépresseurs tricycliques, possède un Number-Needed-to-Treat (NNT, *nombre de sujets à traiter*) plus petit, 3,6 (Saarto and Wiffen, 2007) que celui de la duloxétine, 6,4 (Finnerup et al., 2015). En outre, le temps d'action des antidépresseurs tricycliques, en termes de demi-vie, est beaucoup plus long et leurs cibles secondaires plus variées. Ils peuvent en effet se lier aux canaux ioniques voltages-dépendants (Amir et al., 2006), aux récepteurs histaminiques mais surtout aux récepteurs muscariniques (Pollock et al., 1998; Carnahan et al., 2006), alors qu'aucune propriété anticholinergique n'est connue pour la duloxétine (Carnahan et al., 2006).

Analyse transcriptomique de l'impact de la lésion nerveuse et du traitement.

Nous avons réalisé une analyse transcriptomique afin d'étudier plus en détail le mécanisme d'action périphérique de la duloxétine dans les ganglions rachidiens. Pour cela nous nous sommes placés dans un contexte de maintien de la douleur neuropathique, 5 semaines après la chirurgie et non à un temps court représentant plutôt une phase d'initiation de la douleur.

La lésion nerveuse périphérique entraine un remodelage important de la matrice extracellulaire. En effet, cette lésion provoque en majorité une surexpression de gènes impliqués dans l'organisation tissulaire du ganglion rachidien. Ce phénomène est décrit dans la littérature à travers notamment le rôle des métalloprotéases (MMP) (Kawasaki et al., 2008; Ji et al., 2009) et des intégrines (Dina et al., 2004) dans l'initiation et le maintien de la douleur neuropathique.

Le profil transcriptomique est différent pour l'effet du traitement. Lors d'un traitement prolongé par la duloxétine, on observe une inhibition importante de gènes liés à l'inflammation. Cela concerne en particulier les cytokines et l'inhibition des voies de signalisation NFκB et interféron gamma. Malgré le fait que le traitement a un impact global sur les modifications provoquées par la lésion nerveuse, cette correction est rarement significative si l'on se place au niveau individuel de chaque gène. En effet, il y a peu de gènes

significativement communs entre ceux touchés par le traitement et ceux recrutés par la lésion nerveuse. Par contre, une atténuation partielle de la surexpression ou sous-expression de 80% des gènes liés à la lésion est présente après traitement. La conséquence en est que pour 60% d'entre eux, ils cessent d'être significativement altérés. Par un effet sur la neuroinflammation, la duloxétine atténue ainsi de façon secondaire l'impact à long terme de la lésion nerveuse.

Voie de signalisation TNFα-NFκB.

La voie de signalisation NFκB joue un rôle clé dans la régulation de la production de cytokines pro-inflammatoires ainsi que dans les réponses immunitaires et gliales (Makarov, 2000). Cette même voie de signalisation est présente à la fois en amont de l'induction de cytokines et en aval de la liaison de ces cytokines à leurs récepteurs. En effet, la voie de signalisation NFκB permet la production de TNFα (Ledeboer et al., 2005; Kaltschmidt and Kaltschmidt, 2009) et cette cytokine via sa liaison à son récepteur 1 (TNFR1) active en cascade, en aval, cette même voie de signalisation dans les cellules qui portent le récepteur (Lee et al., 2009). Cette double implication de NFκB peut donc contribuer au maintien de la douleur neuropathique. Les analyses transcriptomiques ont montré un recrutement des gènes appartenant à cette voie, dans les ganglions rachidiens, après une lésion nerveuse périphérique et un abaissement de leur expression après un traitement prolongé par la duloxétine. Ce traitement aboutit également à un effet anti-TNFα. De plus, une inhibition de cette voie NFκB conduit à une normalisation des taux de TNFα et est suffisante pour produire un effet antiallodynique chez l'animal.

Dans cette étude, nous avons montré l'existence d'une dualité d'action de la duloxétine dans un modèle murin de douleur neuropathique périphérique. Les résultats des dosages plasmatiques effectués chez les patients, comparés à ceux effectués chez les animaux, montrent qu'en clinique les taux plasmatiques de duloxétine sont plus proches de ceux obtenus chez les animaux sous traitement prolongé, ce qui suggère que la duloxétine exercerait chez l'homme une action plutôt périphérique dans le soulagement de la douleur neuropathique. En étudiant la plasticité moléculaire conduisant à l'action d'un traitement prolongé sur la neuroinflammation dans les ganglions rachidiens, nous mettons en évidence

un effet anti-neuroimmunitaire de la duloxétine notamment via l'inhibition de la voie de signalisation NFκB-TNFα.

III. δ opioid receptors are essential to the antiallodynic action of β_2-mimetics.

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Introduction

Le système opioïdergique joue un rôle prépondérant dans les contrôles inhibiteurs de la douleur (Mogil et al., 2000). Son implication concerne d'une part l'action directe des analgésiques ciblant les récepteurs MOP (Gaveriaux-Ruff and Kieffer, 2002), telle que la morphine, mais également le recrutement indirect des récepteurs des opioïdes dans l'action des antidépresseurs contre la douleur neuropathique (Marchand et al., 2003; Mico et al., 2006; Benbouzid et al., 2008b). De façon similaire aux antidépresseurs, il a été suggéré que l'action des β_2-mimétiques sur l'allodynie mécanique neuropathique impliquerait le système opioïdergique endogène et plus particulièrement les récepteurs DOP. En effet, une administration aigüe de naltrindole, un antagoniste des récepteurs DOP, supprime ponctuellement l'effet bénéfique du β_2-mimétique clenbutérol dans un modèle de compression du nerf sciatique (Yalcin et al., 2010), ainsi que celui du β_2-mimétique terbutaline dans un modèle de neuropathie diabétique (Choucair-Jaafar et al., 2014). Cette implication des récepteurs DOP dans l'action thérapeutique des agonistes β_2-adrénergiques, ainsi que le rôle éventuel des autres récepteurs des opioïdes, MOP et KOP, restait tout de même à évaluer en utilisant notamment des souris déficientes pour les différents récepteurs des opioïdes.

Avant mon travail sur le sujet, le rôle des récepteurs des opioïdes dans l'action antiallodynique des β_2-mimétiques avait déjà été abordé par les Drs. Yohann Bohren et Salim Megat (travaux non publiés). Par des approches génétiques et pharmacologiques, ils ont montré qu'un traitement prolongé par la terbulatine, administrée par voie intrapéritonéale, a un effet antiallodynique chez les souris sauvages (WT, wild-type) et chez les souris déficientes pour les récepteurs MOP ou KOP. Par contre, ce traitement à la terbutaline perd son effet thérapeutique chez les souris neuropathiques déficientes pour les récepteurs DOP (**Figure 1**). De plus, l'injection d'un antagoniste des récepteurs DOP supprime de façon aigüe l'effet antiallodynique de la terbutaline chez des souris neuropathiques sauvages ou déficientes pour les récepteurs MOP et KOP (**Figure 2A**). Enfin, une administration aigüe de

naloxone méthiodide, un antagoniste des récepteurs des opioïdes, qui ne passe pas la barrière hématoencéphalique, supprime l'effet antiallodynique d'un traitement prolongé par la terbutaline chez des souris neuropathiques sauvages (**Figure 2B**). Ces travaux préliminaires montrent que les récepteurs DOP périphériques sont nécessaires à l'action thérapeutique de la terbutaline, un β_2-mimétique de courte demi-vie. En effet, les β_2-mimétiques sont classés selon deux catégories : 1) les β_2-mimétiques de courte demi-vie, tels que la terbutaline, le salbutamol, le fénotérol et le pirbutérol, à action rapide, possédant une autorisation de mise sur le marché en France pour le traitement curatif des crises d'asthme et de la bronchopneumopathie chronique obstructive ou encore des menaces d'accouchement prématuré ; 2) les β_2-mimétiques de longue demi-vie, tels que le formotérol et le salmétérol, plutôt prescrits à titre préventif de part leur effet prolongé. Il était alors nécessaire de compléter les données préliminaires concernant l'implication des récepteurs des opioïdes dans l'action antiallodynique des β_2-mimétiques en incluant un β_2-mimétique de longue demi-vie, comme le formotérol, mais également en testant une autre voie d'administration que la voie intrapéritonéale utilisée pour la terbutaline, la voie orale via l'eau de boisson.

Methods

Animals

Experiments were performed using C57BL/6J mice (Charles River, L'Arbresle, France) between 8 and 10 weeks-old at the time of surgery, mice lacking MOP, DOP or KOP receptors or mice lacking β_2-AR and their littermate controls. The generation of mice lacking MOP, DOP or KOP receptors has been previously described (Matthes et al., 1996; Simonin et al., 1998; Filliol et al., 2000). The mice lacking β_2-AR were created in the laboratory of Brian Kobilka (Chruscinski et al., 1999). Heterozygote mice were bred in our animal facilities, genotyping of the litters was done upon weaning. Transgenic experiments were conducted on adult male and female (formoterol experiments) or just on male (terbutaline experiments) wild type and knockout littermate mice weighing 20-30 g. When we used male and female, each experimental group contained the same number of males and females. As the wild type animals have the same background and the same behavior, they were pooled to form the control groups. For other experiments, with C57BL/6J mice, we used only males. Mice were group-housed two to five per cage and kept under a 12 hour light/dark cycle with food and water *ad libitum*. A total of 95 C57BL/6J mice, 59 MOP-related, 62 DOP-related, 62 KOP-related and 24 β_2-AR-related transgenic mice were used for the experiments. All animals received proper care in agreement with European guidelines (EU 2010/63). At the end of the experiments, mice were killed by cervical dislocation for immunoblot experiments, or by CO_2 inhalation (CO_2 Euthanasia programmer 6.5 version, TEMSEGA, Pessac, France) followed by cervical dislocation for all other experiments, according to the institutional ethical guidelines. The animal facilities Chronobiotron UMS3415 are registered for animal experimentation under the Animal House Agreement A67-2018-38. All protocols were approved by the "Comité d'Ethique en Matière d'Expérimentation Animale de Strasbourg" (CREMEAS, CEEA35).

Model of neuropathic pain

Neuropathic pain was induced by cuffing the main branch of the right sciatic nerve (Benbouzid et al., 2008c; Yalcin et al., 2014). Surgeries were performed under ketamine (68 mg/kg) / xylazine (10 mg/kg) intraperitoneal (i.p.) anesthesia (Centravet, Tadden, France). The main branch of the right sciatic nerve was exposed and a cuff of PE-20 polyethylene tubing (Harvard Apparatus, Les Ulis, France) of standardized length (2 mm) was unilaterally

inserted around it (Cuff group). The shaved skin was closed using suture. Sham-operated mice underwent the same surgical procedure without implantation of the cuff (Sham group). All mice were allowed to recover from surgery for at least two weeks before starting treatments.

Measure of mechanical allodynia

Mechanical allodynia was tested using von Frey hairs and results were expressed in grams. Tests were done during the morning, starting at least 2 hours after lights on. Mice were placed in clear Plexiglas boxes (7 cm x 9 cm x 7 cm) on an elevated mesh screen. Calibrated von Frey filaments (Bioseb, Vitrolles, France) were applied to the plantar surface of each hindpaw until they just bent, in a series of ascending forces up to the mechanical threshold. Filaments were tested five times per paw and the paw withdrawal threshold (PWT) was defined as the lower of two consecutive filaments for which three or more withdrawals out of the five trials were observed (Barrot, 2012; Yalcin et al., 2014). The person who conducted the tests was blinded to the treatments.

Treatment procedures

The terbutaline treatment began fifteen days after the neuropathy was induced, and it was maintained at least three weeks. During the treatment, the mice received two intraperitoneal injections per day (morning and evening) of terbultaine (0.5 mg/kg, 5 mL/kg, Sigma-Aldrich, St. Quentin Fallavier). Drug was dissolved in 0.9 % NaCl solution that was also used for control injections. The injection of naltrindole (5 mg/kg, subcutaneous, Sigma-Aldrich, St. Quentin Fallavier), a DOP receptor antagonist, was done 36 days after surgery, i.e., after 21 days of terbutaline treatment, in wild-type, MOP and KOP receptor-deficient mice. The injection of naloxone methiodide (5 mg/kg, subcutaneous, Sigma-Aldrich, St. Quentin Fallavier), an opioid receptors antagonist that does not cross the blood-brain barrier, was done following the same procedure as for naltrindole, but in C57BL/6J mice. The mice were von Frey-tested for mechanical sensitivity immediately before the antagonist injections, and 30 min after.

In others experiments, formoterol (BIOTREND Chemicals AG, Zürich, Switzerland) was delivered *per os* through the drinking water with *ad libitum* access as sole source of fluid. The drug was dissolved in water with 0.02% saccharin to increase palatability and control

mice were given a solution of 0.02% saccharin in water (vehicle solution). The long-term formoterol dose-response experiment started two weeks after the surgical procedure (cuff-implantation or sham-surgery). The seven groups (n = 5 per group) consisted: 1) in mice treated with oral formoterol at concentrations 0.5, 0.1, 0.05 or 0.001 µg/mL; 2) sham and cuff control mice treated with the vehicle; 3) another Sham control group that received the highest dose of formoterol (0.5 µg/mL) to test whether formoterol had an analgesic effect per se. Mice were regularly tested for mechanical sensitivity for the whole duration of the dose-response experiment.

Mice lacking MOP, DOP or KOP receptors received formoterol treatment (0.5 µg/mL), in the drinking water, following the same protocol. Data were pooled from four independent experiments, due to the non-regular production of mouse breeding. Four additional sets of mice were also used to pharmacologically assess the role of opioid receptors. Each set was composed of three groups, a sham and a cuff group treated with the oral vehicle, and a cuff group treated with formoterol (0.5 µg/mL). After three weeks of the oral treatment, each of the four sets of mice received a subcutaneous injection of either the MOP receptor antagonist naloxonazine (Sigma-Aldrich, St. Quentin Fallavier, France, 30 mg/kg), of naltrindole, of the KOP receptor antagonist norbinaltorphimine (nor-BNI) (Sigma-Aldrich, St. Quentin Fallavier, 5 mg/kg) or a saline control solution (0.9 % NaCl). All drugs were dissolved in NaCl 0.9%. Mice were von Frey-tested for mechanical sensitivity immediately before injection, and at several time points after.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed with STATISTICA 10 (Statsoft, Tulsa, OK, USA) using multifactor ANOVA. The surgery procedure (Sham or Cuff) and the treatments were taken as between-group factors. When needed, the time of measurement (either time course or preinjection vs. postinjection data) was taken as a within-subject factor. The Duncan test was used for post hoc comparisons. The significance level was set at $p < 0.05$.

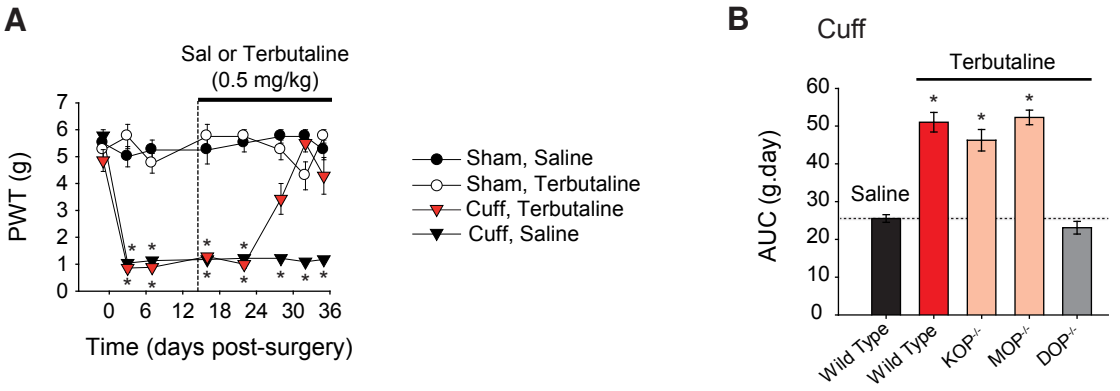

Figure 1. Terbutaline treatment of mechanical allodynia in wild-type, MOP$^{-/-}$, DOP$^{-/-}$ and KOP$^{-/-}$ mice.
Two weeks after unilateral surgery on the right hindpaw, the terbutaline treatment or its saline control solution started and continued until day 36 post-surgery. The mechanical threshold of hindpaw was evaluated using von Frey filaments. (**A**) Chronic terbutaline treatment (0.5 mg/kg, i.p., twice a day) reversed the mechanical allodynia in wild-type mice (n = 8-9 per groups, *p < 0.01 compared to Sham-operated controls receiving Saline). (**B**) Area under the curve (AUC) from post-surgical day 15 (first day of treatment) until the end of treatment for wild-type, MOP$^{-/-}$, DOP$^{-/-}$ and KOP$^{-/-}$ mice. The chronic terbutaline treatment suppressed the ispilateral Cuff-induced allodynia in MOP and KOP receptor-deficient mice but remained ineffective in DOP receptor-deficient mice even after 21 days of treatment (n = 8-12 per groups, *p < 0.01 compared to WT mice receiving Saline). Data are expressed as mean ± SEM.

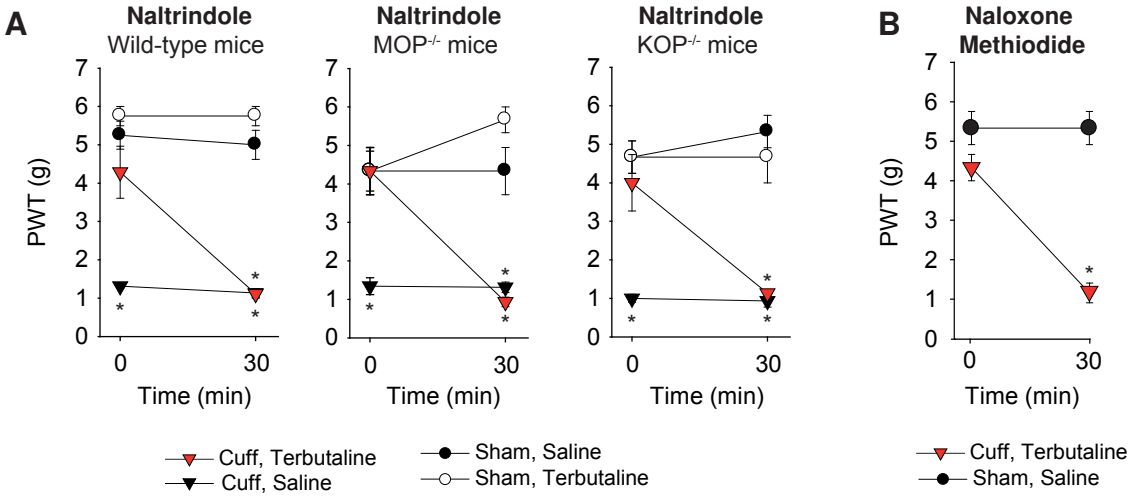

Figure 2. Opioid receptors and terbutaline action.
After 3 weeks of chronic terbutaline treatment (0.5 mg/kg, i.p., twice a day), mice received an acute injection of opioid receptors antagonists. (**A**) An acute administration of naltrindole (5 mg/kg, s.c.), a DOP receptor antagonist, suppressed the antiallodynic effect of terbutaline in wild-type, MOP$^{-/-}$, DOP$^{-/-}$ and KOP$^{-/-}$ mice. (**B**) An acute administration of naloxone methiodide (5 mg/kg, s.c.), an opioid receptor antagonist that does not cross the blood-brain barrier, induced a relapse of the allodynia in treated neuropathic mice. Mice were tested before (0) and 30 min after the acute injection. (n = 6-12 per groups, *p < 0.01 compared to Sham-operated controls receiving Saline). Data are expressed as mean ± SEM.

Results

Terbutaline treatment in opioid receptor deficient mice

As previously reported, cuff-implantation induced an ispsilateral mechanical allodynia in mice (Benbouzid et al., 2008c; Yalcin et al., 2014) (**Figure 1A**; surgery x time interaction, $F_{7,231}$ = 10.25, $p < 0.001$; post hoc: "Cuff Saline" < "Sham Saline" at $p < 0.001$ on post-operative days 4 to 35). Two weeks after Cuff insertion, we started the treatments with either terbutaline (0.5 mg/kg) or the control saline solution (0.9 % NaCl). Mice received two injections per day, 7 days/7, and were tested on given days, in the morning before drug injection. The terbutaline treatment alleviated the cuff-induced allodynia in wild-type mice after about 13 days of treatment (**Figure 1A**; group x time interaction, $F_{7,231}$ = 10.25, $p < 0.001$; post hoc: "Cuff Saline" < "Cuff Terbutaline" at $p < 0.001$ on post-surgery days 28-35). The same antiallodynic effect was also present in MOP (**Figure 1B**; group x time interaction, $F_{3,23}$ = 23.82, $p < 0.001$; post hoc: "Cuff Terbutaline WT" = "Cuff Terbutaline $MOP^{-/-}$" at $p > 0.05$) and KOP receptor-deficient mice (**Figure 1B**; $F_{3,23}$ = 23.82, $p < 0.001$; post hoc: "Cuff Terbutaline WT" = "Cuff Terbutaline $KOP^{-/-}$" at $p > 0.05$), while the terbutaline treatment remained ineffective in DOP receptor-deficient mice (**Figure 1B**; group x time interaction, $F_{3,23}$ = 23.82, $p < 0.001$; post hoc: "Cuff Terbutaline $DOP^{-/-}$" < "Cuff Terbutaline WT" at $p < 0.001$).

Effect of the DOP receptor antagonist naltrindole on terbutaline antiallodynic action

We tested the consequences of an acute injection with the DOP receptor antagonist naltrindole (5 mg/kg) in the wild-type, MOP and KOP receptor-deficient mice. After 3 weeks of treatment with terbutaline or saline, the acute injection of naltrindole totally suppressed the benefit of terbutaline treatment. Within 30 min following the DOP receptor antagonist injection, we observed a relapse of allodynia. This effect was present both in wild-type, MOP and KOP receptor-deficient mice (**Figure 2A**; group x time interaction, WT: $F_{1,33}$ = 18.83, $p < 0.001$; post hoc: "Cuff Terbutaline WT" < "Sham Terbutaline WT" at $p < 0.001$ on time 30 min; $MOP^{-/-}$: $F_{1,21}$ = 21.61, $p < 0.001$; post hoc: "Cuff Terbutaline $MOP^{-/-}$" < "Sham Terbutaline $MOP^{-/-}$" at $p < 0.01$ on time 30 min; $KOP^{-/-}$: $F_{1,20}$ = 5.05, $p < 0.01$; post hoc: "Cuff Terbutaline $KOP^{-/-}$" < "Sham Terbutaline $KOP^{-/-}$" at $p < 0.01$ on time 30 min). We also

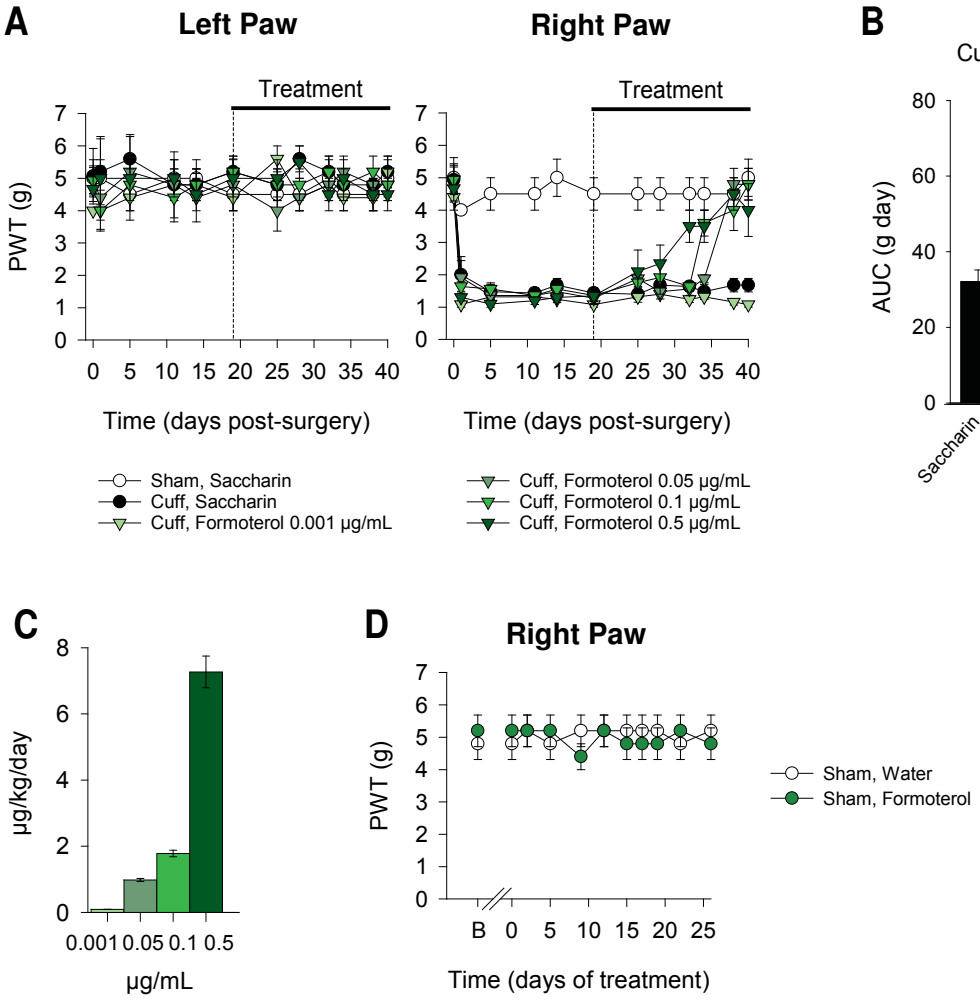

Figure 3. Chronic oral formoterol treatment suppresses the cuff-induced sustained mechanical allodynia.
Two weeks after unilateral surgery on the right hindpaw, the chronic oral treatment with formoterol started and lasted 21 days. **(A)** The animals (n = 5 per group) were administered formoterol (0.001, 0.05, 0.1 or 0.5 µg/mL) with saccharin 0.02% or saccharin alone in the drinking water as the sole source of fluid. The mechanical sensitivity (paw withdrawal threshold, PWT) was evaluated over days using von Frey filaments. The vehicle treatment (Saccharin 0.02%) did not affect the mechanical sensitivity of either Sham or Cuff mice. Chronic oral formoterol treatment at the three highest doses reversed the cuff-induced allodynia. The treatment at dose of 0.001 µg/mL had no effect. **(B)** Area under the curve (AUC) from for post-surgical day 19 (first day of treatment) until the end of the treatment for formoterol (0.001, 0.05, 0.1 or 0.5 µg/mL). *p < 0.05 compared to Cuff-operated control group drinking vehicle. **(C)** Histogram showing the equivalence between µg/mL and µg/kg/day of the different doses. **(D)** Formoterol treatment (0.5 µg/mL)did not affect mechanical sensitivity of sham-operated mice. Data are expressed as mean ± SEM.

controlled that naltrindole per se had no effect in mice with Sham surgery, or in neuropathic mice treated with saline.

Peripherals opioid receptors are involved in terbutaline antiallodynic effect

The acute injection of naloxone methiodide (5 mg/kg), an opioid receptor antagonist that does not cross the blood-brain barrier, induced a relapse of allodynia in Cuff-implanted mice treated with terbutaline. This effect was present 30 min after the acute administration (**Figure 2B**; group x time interaction, $F_{1,20} = 10.9$, $p < 0.01$; post hoc: "Cuff Terbutaline" < "Sham Saline" at $p < 0.001$ on time 30 min). The same dose of naloxone methiodide had no effect in mice with Sham surgery treated with saline.

Antiallodynic action of chronic oral formoterol: dose response

The mechanical sensitivity of mice was assessed using von Frey hairs. Although sham surgery did not influence mechanical thresholds (**Figures 3A,D**), cuff implantation induced an ipsilateral mechanical allodynia (**Figure 3A**; surgery x time interaction, $F_{11,242} = 5.8$, $p < 0.001$; post hoc: "Cuff Saccharin" < "Sham Saccharin" at $p < 0.001$ on all post-operative days). We did not observe any change in the nociceptive threshold on the left paw. Nineteen days after surgery, we started treatment with different doses of formoterol (0.001, 0.05, 0.1 or 0.5 µg/mL) or with saccharin vehicle solution (0.02% saccharin). Formoterol treatment at doses 0.05, 0.1 and 0.5 µg/mL alleviated the cuff-induced allodynia after nineteen days, fifteen days and thirteen days of treatment for 0.05, 0.1 and 0.5 µg/mL respectively (**Figure 3A**; group x time interaction, $F_{11,242} = 5.8$, $p < 0.001$; post hoc: "Cuff Saccharin" < "Cuff Formoterol 0.05 µg/mL" at $p < 0.001$ on post-surgery days 38-40, "Cuff Saccharin" < "Cuff Formoterol 0.1 µg/mL" at $p < 0.001$ on post-surgery days 34-40, "Cuff Saccharin" < "Cuff Formoterol 0.5 µg/mL" at $p < 0.001$ on post-surgery days 32-40). The 0.001 µg/mL dose of formoterol had no significant effect. Treatments at different doses did not affect the contralateral nociceptive thresholds (Left Paw, **Figure 3A**). The dose response effect of formoterol can also be visual with data presented as area under the curve (AUC) (**Figure 3B**).

The drinking bottles were regularly weighed during the experiment. Considering the volume of solution drank by the mice per 24 h, the 0.001 µg/mL solution was equivalent to 0.2 ± 0.01 µg/kg/day, the 0.05 µg/mL solution was equivalent to 0.98 ± 0.05 µg/kg/day, the 0.1 µg/mL solution was equivalent to 1.78 ± 0.10 µg/kg/day and the 0.5 µg/mL solution was

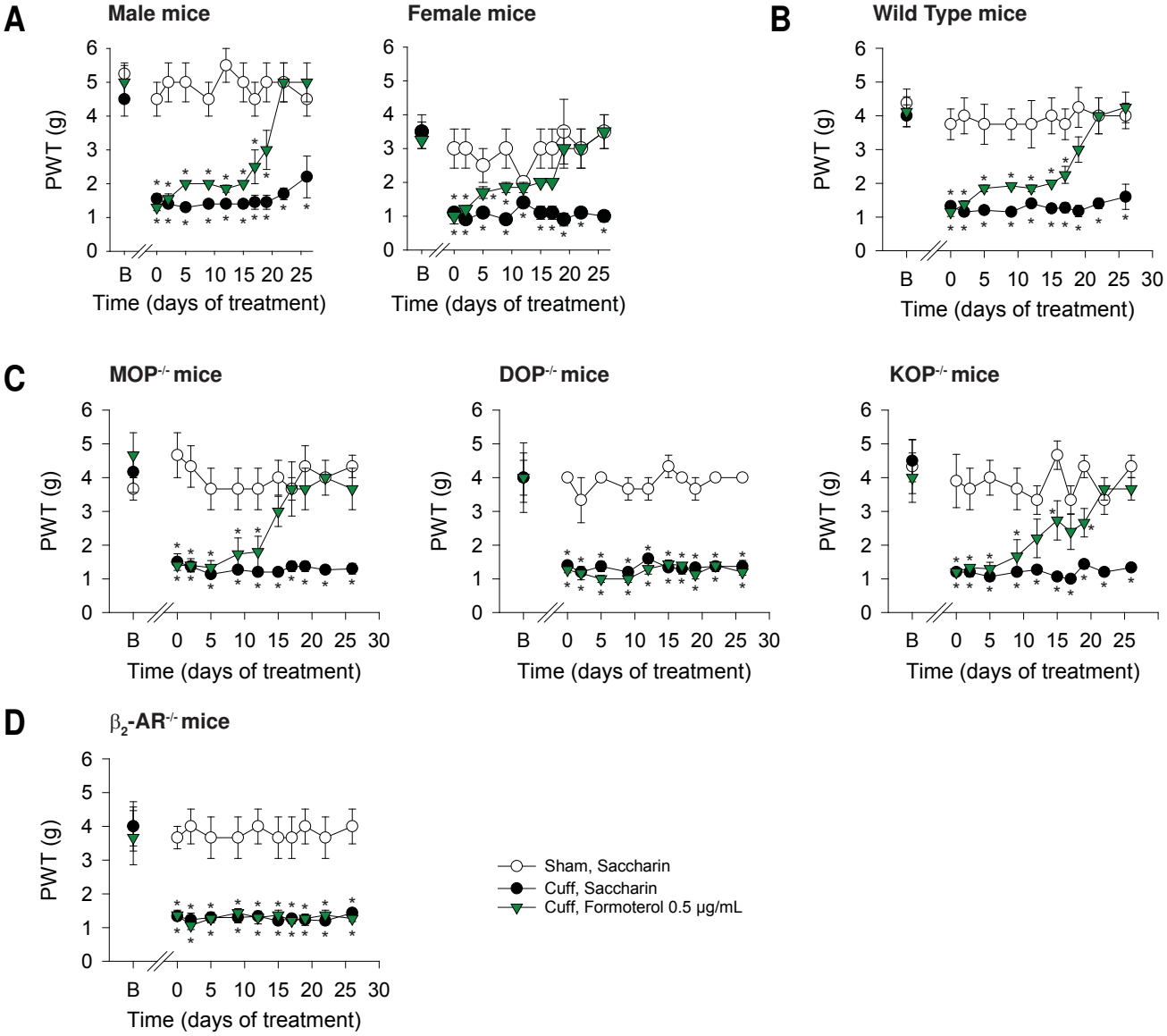

Figure 4. Effect of chronic oral formoterol treatment on mechanical sensitivity of the right paw in wild type and β_2-AR-, MOP-, DOP- or KOP-deficient mice.

Between two and five weeks after Cuff implantation, the oral treatment with formoterol (0.5 µg/ml) or its saccharin 0.02% solution control started and was maintained for over 3 weeks. Mechanical allodynia was tested using von Frey filaments. (**A**) The mechanical sensitivity threshold (PWT) of female mice is lower than that of male mice. However, both sexes developed mechanical allodynia similarly and formoterol was effective in reversing the cuff-induced allodynia in both male and female mice. Males and females were then pooled in each experimental group. (**B**) Chronic formoterol treatment abolishes the ipsilateral cuff-induced allodynia in wild-type mice, as well as in MOP or KOP receptor-deficient mice, but remains ineffective in DOP receptor-deficient mice (**C**). (**D**) The mechanical allodynia is no more affected by the chronic formoterol treatment in β_2-AR deficient mice. (n = 6-8 males and females per group, $^*p < 0.05$ compared to Sham-operated control group drinking vehicle). Data are expressed as mean ± SEM.

equivalent to 7.27 ± 0.48 µg/kg/day (**Figure 3C**). These amounts were in fact mostly taken over the 12 h night period, period during which mice usually drink.

To test if formoterol have an analgesic effect, we evaluate the higher dose of formoterol (0.5 µg/mL) on sham control mice. Chronic oral treatment with 0.5 µg/mL did not affect mechanical thresholds of mice of the Sham group (**Figure 3D**).

Chronic oral formoterol treatment in opioid and beta2-adrenergic receptor deficient mice

Mechanical sensitivity thresholds of female mice were significantly lower than in males (baseline threshold values of right paws are equal to 3.4 g ± 0.7 for females and 4.9 g ± 0.9 for males, females vs. males: $t_{11} = 4.18$, $p < 0.005$). Both male and female mice developed mechanical allodynia after surgery, and formoterol treatment suppressed the cuff-induced allodynia in both sexes (**Figure 4A**; Female mice: group x time interaction, $F_{10,90} = 2.2$, $p < 0.01$; post hoc: "Cuff Saccharin" < "Cuff Formoterol" at $p < 0.005$ on treatment days 19-26; Male mice: group x time interaction, $F_{10,90} = 5.4$, $p < 0.001$; post hoc: "Cuff Saccharin" < "Cuff Formoterol" at $p < 0.001$ on treatment days 22-26).

MOP-, DOP-, KOP-receptors or β_2-AR deficient mice displayed the same baseline for mechanical sensitivity as their wild-type littermates. Two weeks after the cuff insertion, we started the oral treatment with either formoterol (0.5 µg/mL) or the control solution (0.02 % saccharin). Formoterol treatment alleviated cuff-induced allodynia in wild-type mice (**Figure 4B**; group x time interaction, $F_{10,210} = 5.6$, $p < 0.001$; post hoc: "Cuff Saccharin" < "Cuff Formoterol" at $p < 0.05$ on post-treatment day 19 and at $p < 0.001$ on post-treatment days 22-26). The same antiallodynic effect was also present in MOP (**Figure 4C**; group x time interaction, $F_{10,150} = 5.5$, $p < 0.001$; post hoc: "Cuff Saccharin" < "Cuff Formoterol" at $p < 0.05$ on treatment day 15 and at $p < 0.005$ on treatment days 17-26) and KOP receptor-deficient mice (**Figure 4C**; group x time interaction, $F_{10,150} = 4.1$, $p < 0.001$; post hoc: "Cuff Saccharin" < "Cuff Formoterol" at $p < 0.05$ on treatment day 15 and at $p < 0.001$ on treatment days 22-26), while the formoterol treatment remained ineffective in DOP receptor-deficient mice (**Figure 4C**; group x time interaction, $F_{10,150} = 2.7$, $p < 0.001$; post hoc: "Cuff Formoterol" < "Sham Saccharin" at $p < 0.001$ on treatment days 0-26) and in β_2-AR deficient mice (**Figure 4D**; group x time interaction, $F_{10,150} = 1.8$, $p < 0.05$; post hoc: "Cuff Formoterol" < "Sham Saccharin" at $p < 0.001$ on treatment days 0-26).

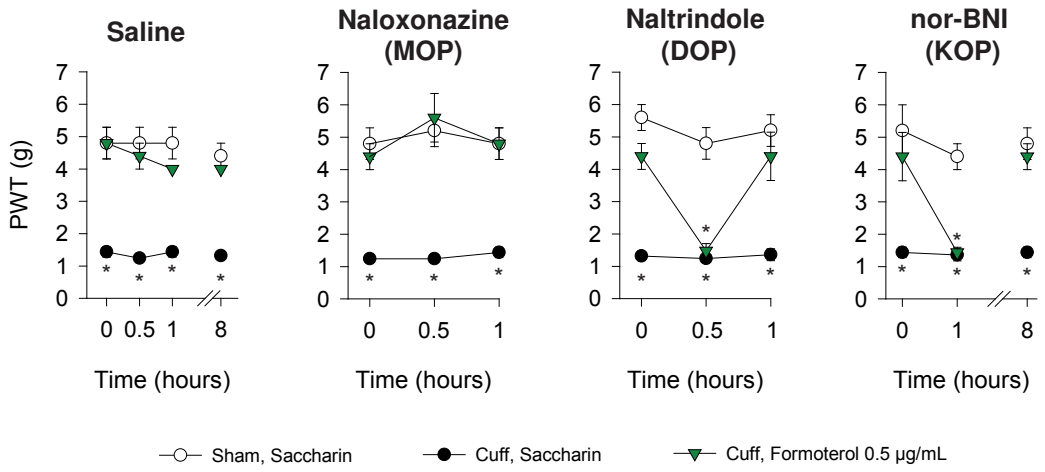

Figure 5. Role of opoid receptors in the antiallodynic effect of formoterol.
After at least 3 weeks of formoterol or saccharin treatment, the animals received an injection of the MOP receptor antagonist naloxonazine (30 mg/kg, s.c.), DOP receptor antagonist naltrindole (5 mg/kg, s.c.), KOP receptor antagonist nor-BNI (5 mg/kg, s.c.) or their control saline solution. The mechanical threshold was measured before, 30 min and 1 h after acute injection for naloxonazine and naltrindole. For nor-BNI, a slow-acting antagonist, mechanical threshold was measured before, 1 h and 8 h after injection. Naltrindole and nor-BNI induced a transitory relapse of mechanical allodynia in neuropathic mice treated by formoterol. Naloxonazine was no effect on formoterol treated-mice. (Data are expressed as mean ± SEM, n = 5 per group, *$p < 0.001$ compared to Sham-operated control group drinking vehicle).

Opioid receptor antagonists effect on long lasting formoterol treatment

We tested the consequences of an acute injection with the MOP receptor antagonist naloxonazine, or with the DOP receptor antagonist naltrindole, or with the KOP receptor antagonist nor-BNI. The naloxonazine and the naltrindole are fast-acting antagonists while the nor-BNI is selective of KOP receptors only 8 hours after the acute injection, 1 hour after the injection it also acts on MOP receptors (Megat et al., 2015). After 3 weeks of treatment with formoterol or saccharin, the acute injection of naltrindole totally suppressed the benefit of formoterol treatment (**Figure 5**; group x time interaction, $F_{2,24} = 5.3$, $p < 0.005$; post hoc: "Cuff Formoterol" < "Sham Saccharin" at $p < 0.001$ on time 30 min). Similarly, the injection of nor-BNI attenuated the formoterol-induced relapse of allodynia 1 hour after the acute administration (**Figure 5**; group x time interaction, $F_{2,24} = 4.4$, $p < 0.01$; post hoc: "Cuff Formoterol" < "Sham Saccharin" at $p < 0.001$ on time 1 h) but these effect disappeared 8 hours later (Figure 5; group x time interaction, $F_{2,24} = 4.4$, $p < 0.01$; post hoc: "Cuff Saccharin" < "Cuff Formoterol" at $p < 0.001$ on time 8 h). Naloxonazine did not suppress the antiallodynic action of formoterol treatment. We also observed that naloxonazine, naltrindole or nor-BNI per se had no effect in mice with Sham surgery or in mice that received saccharin alone.

Discussion

Par une approche transgénique et pharmacologique nous avons évalué le rôle des récepteurs des opioïdes dans l'action antiallodynique de deux β_2-mimétiques, l'un de courte demi-vie, la terbutaline, administrée par voie intrapéritonéale et l'autre de longue demi-vie, le formotérol, administré par voie orale. Les résultats montrent qu'une concentration faible de formotérol par voie orale ou de terbutaline par voie intrapéritonéale est suffisante pour induire un effet antiallodynique chez l'animal, et ce après un délai thérapeutique similaire à celui d'autres β_2-mimétiques (Yalcin et al., 2010) ou à celui des antidépresseurs, soit environ 13 jours (Yalcin et al., 2009b; Yalcin et al., 2010). Grâce à l'utilisation d'animaux transgéniques, nous avons montré que les récepteurs DOP, mais pas les récepteurs MOP ou KOP, étaient nécessaires à l'action thérapeutique des β_2-mimétiques testés, la terbutaline et le formotérol. De plus, par une approche pharmacologique, nous montrons que les récepteurs DOP impliqués seraient préférentiellement périphériques.

Nos résultats montrent que quelque soit leur demi-vie la terbutaline et le formotérol exercent leurs effets avec un délai thérapeutique identique, d'environ 13 jours, équivalent à celui des antidépresseurs (Choucair-Jaafar et al., 2009; Yalcin et al., 2009a; Yalcin et al., 2009b; Yalcin et al., 2010; résultats présents). En outre, le délai thérapeutique ne semble pas dépendre, ou très peu, des doses utilisées (Benbouzid et al., 2008a; Choucair-Jaafar et al., 2009). Le délai d'action de ces traitements dans le cadre de la douleur neuropathique est donc indépendant des propriétés pharmacocinétiques des molécules et des voies d'administration ; par contre, il semble lié à la mise en place de phénomènes de neuroplasticité moléculaire et cellulaire (Barrot et al., 2009), comme cela a pu être décrit avec les antidépresseurs dans le cadre de la dépression (Duman, 2002; Vialou et al., 2013; Rantamaki and Yalcin, 2016).

Nous avons également testé une voie d'administration peu utilisée en préclinique mais majoritaire en clinique, l'administration par voie orale, pour le formotérol. L'administration via l'eau de boisson de ce β_2-mimétique présente l'avantage d'être non invasif et moins stressant que les administrations intrapéritoénales répétées. En effet, des études ont montré que des injections répétées d'antidépresseurs, chez la souris, peuvent induire un état de stress chronique (Caldarone et al., 2003). De même, chez le rat des injections répétées de solution saline entrainent un comportement dépressif (Izumi et al.,

1997). Enfin, l'administration par voie orale via l'eau de boisson permet de se rapprocher d'avantage de la posologie humaine et de la prise orale d'un médicament.

Les β_2-mimétiques restent efficaces chez les animaux déficients pour les récepteurs MOP ou KOP mais perdent leur efficacité chez les souris déficientes pour les récepteurs DOP. Les récepteurs DOP seraient donc les seuls récepteurs des opioïdes nécessaires à l'action antiallodynique des β_2-mimétiques. Les données pharmacologiques soutiennent ces résultats. En effet, l'injection aigüe de naltrindole, un antagoniste de ces récepteurs, entraine une réapparition transitoire de l'allodynie mécanique chez des souris préalablement soulagées de ce symptôme par les β_2-mimétiques. Il convient toutefois de remarquer qu'une administration aigüe de norbinaltorphimine (nor-BNI), antagoniste des récepteurs KOP, bloque l'effet antiallodynique du formotérol 60 minutes après son administration, alors qu'à plus long terme, l'action antiallodynique du β_2-mimétique est conservée. Cette contradiction avec les résultats obtenus chez les animaux transgéniques s'explique par le manque de sélectivité de la nor-BNI dans les premières heures suivant son injection (Endoh et al., 1992; Megat et al., 2015). En effet, cet antagoniste peut toucher les autres récepteurs des opioïdes, dont les récepteurs DOP, durant les premières heures suivant son administration (Endoh et al., 1992; Megat et al., 2015), expliquant ainsi le blocage de l'effet antiallodynique du formotérol.

Ces résultats sont à rapprocher des résultats obtenus précédemment montrant le rôle crucial des récepteurs DOP dans l'action antiallodynique des antidépresseurs (Benbouzid et al., 2008b; *cf. p.94*). Alors que les β_2-AR et les récepteurs DOP sont tous deux nécessaires à l'action antiallodynique des antidépresseurs tricycliques (Benbouzid et al., 2008a; Benbouzid et al., 2008b; Yalcin et al., 2009a; Yalcin et al., 2009b) et des β_2-mimétiques (Choucair-Jaafar et al., 2009; Yalcin et al., 2010), le lien mécanistique entre ces deux récepteurs reste incertain. Fait intéressant, la participation de ces deux récepteurs a également été observée dans l'effet antinociceptif induit par une administration intraplantaire d'un agoniste β-AR suite à une douleur inflammatoire (Binder et al., 2004) ainsi que dans l'action antiallodynique d'un traitement prolongé par le curcuma dans un modèle de constriction du nerf sciatique (Zhao et al., 2012). De plus, des évidences existent quant à la présence de ces deux récepteurs, β_2-AR et DOP, au sein du système nociceptif périphérique. Les récepteurs DOP sont exprimés par des fibres non-peptidergiques myélinisées de type Aβ, alors que les récepteurs MOP sont présents sur les petites fibres

peptidergiques non-myélinisées de type C (Scherrer et al., 2009). Toutefois ce point de vue est débattu (Gendron et al., 2015) étant donné que les récepteurs DOP sont également largement exprimés sur les corps cellulaires des neurones Aβ et C mais également Aδ (Wang et al., 2010). Par contre, ces récepteurs des opioïdes sont affectés dans les modèles de neuropathie périphérique car au site de lésion et dans les ganglions rachidiens, la neuropathie conduit à une surexpression des récepteurs DOP (Kabli and Cahill, 2007). Les β_2-AR seraient quant à eux préférentiellement exprimés par les cellules satellites gliales des ganglions rachidiens (Elenkov et al. 2000; Bohren et al., 2013 *cf. p.16)*. Ces cellules non neuronales sont des analogues périphériques des astrocytes du système nerveux central et qui sont connues pour exprimer les β_2-AR à leur membrane dans diverses espèces incluant l'homme (Trimmer et al., 1984; Mantyh et al., 1995). Comme pour les récepteurs DOP, une augmentation de l'expression des β_2-AR est observée dans les ganglions rachidiens suite à une lésion du nerf sciatique (Maruo et al., 2006).

Les récepteurs DOP ne sont cependant pas les cibles directes d'un traitement par des antidépresseurs ou des β_2-mimétiques et il est nécessaire afin d'élucider le mécanisme d'antiallodynie exercé par un β_2-mimétique, de comprendre les liens cellulaire et moléculaire entre la stimulation du système adrénergique et le recrutement du système opioïdergique endogène. Plusieurs hypothèses peuvent être soulevées :

1) Les β_2-AR et les récepteurs DOP font parti d'une même cascade. En effet, le délai thérapeutique d'une quinzaine de jours et la rechute immédiate après une administration aigüe de naltrindole suggèrent que la composante opioïdergique est probablement située en aval de la composante adrénergique. D'autre part, nous avons également montré qu'une administration aigüe de naloxone méthiodide supprimait l'effet antiallodynique d'un traitement prolongé par la terbulatine, ce qui laisse penser que l'action du système opioïdergique a lieu en périphérie, dans le nerf ou le ganglion rachidien. Ainsi, nous pouvons émettre l'hypothèse que dans les ganglions rachidiens, le recrutement des β_2-AR (Bohren et al., 2013 *cf. p.16)*, indirectement par les antidépresseurs via le système nerveux sympathique ou directement par un β_2-mimétique, conduit à l'activation de la voie AMPc/PKA dans les cellules satellites gliales qui mènerait à la synthèse et à la libération de peptides opioïdes comme les enképhalines, lesquels stimuleraient les récepteurs DOP présents sur les afférences primaires (Binder et al., 2004; Busch-Dienstfertig and Stein, 2010).

2) Les β_2-AR et les récepteurs DOP sont co-exprimés par les cellules satellites gliales. En effet, il a pu être observé, dans des cultures de cellules transfectées, l'existence de complexes hétéromères de récepteurs DOP et β_2-AR à la membrane (Jordan et al., 2001; Ramsay et al., 2002; Cao et al., 2005). Cependant, il n'y a encore aucune évidence que les deux récepteurs puissent être co-exprimés par les mêmes cellules au sein du système nociceptif. De plus, même s'il a été montré que les β_2-AR sont principalement exprimés par les cellules satellites gliales (Elenkov et al., 2000; Bohren et al., 2013), les récepteurs DOP semblent quant à eux majoritairement exprimés sur les afférences primaires (Mennicken et al., 2003), ce qui rend peu probable cette deuxième hypothèse.

3) les récepteurs DOP font partie d'un mécanisme « permissif », dans lequel leur présence est indispensable au fonctionnement normal des voies responsables de l'action antiallodynique, sans pour autant qu'ils soient un élément de la cascade moléculaire responsable de cette action. Ceci pourrait être étayé par le fait que les récepteurs DOP seraient plutôt impliqués dans les réponses nociceptives mécaniques que thermiques (Scherrer et al., 2009). Cependant d'autres recherches sont nécessaires pour élucider cette question.

En conclusion, cette étude a permis de montrer que les récepteurs DOP périphériques étaient essentiels à l'action antiallodynique des β_2-mimétiques dans un modèle de compression du nerf sciatique chez la souris. Antidépresseurs et β_2-mimétiques sembleraient ainsi recruter les mêmes effecteurs secondaires suite à la stimulation des β_2-AR. D'autres études sont cependant nécessaires pour élucider le lien mécanistique entre ces deux récepteurs.

DISCUSSION GÉNÉRALE

Dans ce travail de thèse, nous avons étudié les effets thérapeutiques de traitements de la douleur neuropathique périphérique dans un modèle murin. Nous montrons que le système opioïdergique n'est pas nécessaire à l'action antiallodynique des gabapentinoïdes, mais que cette action agit sur une composante neuroimmunitaire de la douleur neuropathique. Nous mettons également en évidence une dualité de mécanisme d'action de la duloxétine, selon la dose administrée et la durée du traitement. Ainsi, à doses faibles à moyennes, après traitement chronique, elle permet un soulagement prolongé de la douleur neuropathique en recrutant la noradrénaline du système sympathique périphérique, qui agit via les β_2-AR pour bloquer l'expression du TNFα. Le mécanisme implique également les récepteurs DOP périphériques. La plasticité moléculaire de ce mécanisme a également été étudiée par des techniques de séquençage d'ARN. Par contre, à doses plus fortes, la duloxétine exerce une action antalgique centrale, en recrutant la noradrénaline des voies descendantes inhibitrices qui agit alors via les α_{2A}-AR. Ce mécanisme implique les récepteurs MOP et DOP centraux. En clinique, les résultats des dosages des taux plasmatiques de duloxétine laissent supposer que la duloxétine exercerait une action antalgique plutôt périphérique que centrale. Enfin, nous montrons que les agonistes des β_2-AR et les antidépresseurs partagent une cible commune pour soulager l'allodynie neuropathique, les récepteurs DOP.

Certains aspects des résultats sont discutés dans les articles présentés. Nous ne reviendrons pas dessus dans cette discussion générale. Nous reprendrons ici trois grandes questions évoquées dans les articles afin de les discuter plus en profondeur, dans une vision principalement mécanistique. Seront abordés les points suivants : **I)** le rôle de la neuroinflammation dans la douleur neuropathique et l'action des traitements sur cette composante ; **II)** l'implication du système noradrénergique dans la douleur neuropathique et ses traitements ; **III)** la validité de la recherche translationnelle et ses limites.

I. Douleur neuropathique et neuroinflammation

1. Définition de la neuroinflammation

Avant de parler de « neuroinflammation » ou d'« inflammation neurogène » les scientifiques utilisaient le terme de « gliose réactive » (Norton et al., 1992; Streit et al., 1999) pour décrire les réponses endogènes des tissus du système nerveux survenant suite à des blessures. Ce terme faisait référence à l'accumulation, rapidement après une lésion du système nerveux central, de cellules gliales, notamment des astrocytes, et de la microglie. Mais ce terme suggère une passivité des cellules gliales face à la lésion, or ces cellules libèrent des facteurs qui agissent et modulent les réponses des cellules cibles, réponses analogues à celles que l'on retrouve au sein du système immunitaire. Ces facteurs vont conduire à l'activation des cellules immunitaires à la périphérie et à l'infiltration de cellules immunocompétentes (macrophages, lymphocytes…) dans les tissus (Marchand et al., 2005; Kim and Moalem-Taylor, 2011). La neuroinflammation est donc caractérisée par l'activation des cellules gliales, la libération accrue de facteurs inflammatoires, associées ou non au recrutement de cellules immunocompétentes. Ces facteurs mis en jeu au cours de la neuroinflammation sont des messagers chimiques et principalement des cytokines pro- et anti-inflammatoires, des chimiokines et des prostaglandines.

Les cytokines sont des protéines immunoréactives secrétées par les polynucléaires neutrophiles, les macrophages et les lymphocytes, ainsi que par les cellules gliales, qui peuvent agir sur les neurones via des récepteurs spécifiques (Old et al., 2015). Le TNFα figure parmi les cytokines pro-inflammatoires les mieux étudiées, mais d'autres sont également importantes lors de la neuroinflammation, telles que les interleukines, IL-1β et IL-6 pro-inflammatoires ou encore IL-10 et IL-4 anti-inflammatoires (Austin and Moalem-Taylor, 2010).

2. Rôle de la neuroinflammation dans la douleur neuropathique

a. Cellules immunitaires et cellules satellites

Suite à une lésion nerveuse périphérique, on observe un recrutement et une invasion de cellules immunocompétentes dans le ganglion rachidien ; cellules normalement peu

présentes dans les conditions basales (Calvo et al., 2012). On décrit notamment un recrutement de macrophages et de lymphocytes (Lu and Richardson, 1993; Sommer and Schafers, 1998; Hu and McLachlan, 2002) en réponse à des molécules chimioattractantes libérées par les cellules gliales (Campana, 2007), ainsi qu'une augmentation des polynucléaires neutrophiles (Morin et al., 2007) et des cellules dendritiques (Kim and Moalem-Taylor, 2011).

Dans le modèle de compression du nerf sciatique utilisé, aucune donnée n'existe quant au recrutement ou à l'implication des cellules immunocompétentes dans l'initiation ou le maintien de l'allodynie mécanique. Par contre, nous avons décrit un rôle potentiel des cellules satellites. En effet, par qPCR et Western blot, nous montrons une augmentation des taux de mTNFα dans les ganglions rachidiens associée à la présence d'une allodynie mécanique, 5 semaines après la pose du manchon comprimant le nerf sciatique. Par immunohistochimie, nous avons ensuite montré que ce TNFα était principalement exprimé par les cellules non neuronales du ganglion rachidien, les cellules satellites, et qu'un traitement par immunothérapie anti-TNFα soulageait l'allodynie.

Les cellules satellites enveloppent les neurones sensoriels des ganglions rachidiens (Hanani, 2005). Après lésion nerveuse périphérique, une activation et une prolifération (Shinder et al., 1999) de ces cellules, se traduisant par une augmentation d'expression de la Glial Fibrillary Acidic Protein (GFAP, *protéine acide fibrillaire gliale*) (Ohtori et al., 2004) et de la protéine S100 (Sandelin et al., 2004), se produit dans les ganglions rachidiens. Dans notre modèle, l'analyse génomique met en avant un remodelage important, et à long terme, de la matrice extracellulaire, ainsi qu'une implication de métalloprotéases (MMP) qui participent au développement de la douleur neuropathique (Ji et al., 2009). Exprimées par les neurones des ganglions rachidiens mais également par les cellules satellites, elles réguleraient la prolifération et la différenciation des cellules gliales périphériques (Chattopadhyay and Shubayev, 2009; Berta et al., 2012) et centrales (Kawasaki et al., 2008). Ces données soulignent un lien étroit entre matrice extracellulaire, cellules gliales et immunocompétentes, et neuroinflammation. Une étude menée dans un modèle de douleur induite par la morphine avance même que la MMP-9 serait le signal d'activation des cellules satellites gliales (Berta et al., 2012). Dans notre modèle, l'expression de la MMP-9 ne semble pas modifiée, par contre celle de MMP-16 est significativement augmentée après lésion nerveuse périphérique. Peu de données existent quant au rôle de cette MMP dans la

douleur, mais dans d'autres pathologies, et notamment le cancer, elle est impliquée dans l'invasion de cellules immunocompétentes dans les tissus tumoraux (Tatti et al., 2015; Wang et al., 2015a). Il est également à noter que l'expression de MMP-9, bien que non modifiée chez l'animal neuropathique, est significativement réduite par le traitement prolongé par la duloxétine.

Enfin, la neuroinflammation ne se limite pas aux seuls ganglions rachidiens. On retrouve ce processus au site de lésion mais également au niveau spinal où les cellules microgliales activées expriment un récepteur ionotropique des purines, le P2X4, dont l'activation conduit à une hyperexcitabilité neuronale via la libération accrue du Brain-Derived Neurotrophic Factor (BDNF, *facteur neurotrophique dérivé du cerveau*) par ces cellules (Tsuda et al., 2003). Ce facteur neurotrophique serait responsable d'interactions neurone-glie favorisant une hyperexcitabilité neuronale (Trang et al., 2006) en changeant le potentiel d'équilibre anionique, réduisant ainsi l'effet de l'ouverture des canaux chlorure par la glycine et le GABA (Coull et al., 2005). La chimiokine Monocyte Chemoattractant Protein-1 (MCP-1, *protéine 1 chimioattractive monocytaire*), dont l'expression est augmentée dans les ganglions rachidiens et dans les terminaisons centrales des neurones sensoriels après une lésion nerveuse périphérique, servirait de signal d'activation microgliale dans la corne dorsale de la moelle épinière (Zhang and De Koninck, 2006). Ceci suggère l'existence d'une cascade d'activation neuroimmunaitaire, démarrant à la périphérie, au niveau du site de lésion puis dans le ganglion rachidien entraînant ensuite des phénomènes centraux. Récemment, une étude a montré que ce mécanisme neuroimmunitaire, décrit chez les souris mâles, pouvait être différent chez les femelles. Chez celles-ci, l'immunité adaptative via l'infiltration de lymphocytes T serait importante pour l'effet allodynique de la lésion nerveuse (Sorge et al., 2015). Ces résultats soulèvent donc la question d'une différence de mécanisme neuroimmunitaire selon le sexe. Il était donc important pour nous de montrer l'effet du traitement à fois chez les souris mâles et femelles, afin de détecter une éventuelle différence mécanistique. Nos résultats montrent qu'un traitement prolongé par la duloxétine possède un effet anti-TNFα dans les ganglions rachidiens chez les deux sexes, ce qui n'exclut pas l'existence de voies différentes conduisant au même effet.

b. Cytokines pro- et anti-inflammatoires

Nos résultats obtenus par Western blot mettent en évidence une augmentation durable des taux de mTNFα dans les ganglions rachidiens ispilatéraux à la pose du manchon. Ceci a également été confirmé par qPCR (Bohren et al., 2013). Le TNFα existe sous deux formes biologiquement actives, une forme membranaire de 26 kDa, clivée en une forme soluble de 17 kDa par une MMP, l'enzyme de conversion du TNFα (TACE) (Horiuchi et al., 2010). Ces deux formes se fixent aux récepteurs 1 (TNFR1) et 2 (TNFR2) du TNF, qui sont membranaires mais peuvent, par clivage protéolytique, également exister sous forme soluble permettant de moduler la biodisponibilité du TNFα (Opal and DePalo, 2000).

L'expression du TNFα peut être corrélée au développement d'une hyperalgésie ou d'une allodynie chez le patient neuropathique (Empl et al., 2001; Uceyler et al., 2007), mais également dans divers modèles animaux de douleur neuropathique (Leung and Cahill, 2010; Wang et al., 2015b). Comme dans notre modèle, une augmentation des taux de TNFα dans les ganglions rachidiens est aussi décrite après lésion du nerf sciatique (Ohtori et al., 2004), ou après application de nucleus polpusus sur une racine nerveuse (Handa et al., 2016). Dans d'autres modèles, les résultats diffèrent en partie. Ainsi, après ligature du nerf sciatique, un retour à l'état basal de l'ARNm du TNFα a été observé dès 7 jours post-chirurgie (Lee et al., 2004; Sacerdote et al., 2008). Par contre, une augmentation de mTNFα est bien présente dans les ganglions rachidiens 21 jours après lésion de la racine nerveuse L5 (Hatashita et al., 2008). On peut ainsi imaginer une implication du TNFα dans une période précoce de l'allodynie, liée principalement au geste chirurgical, mais également dans une période plus tardive où cette cytokine serait un acteur important dans le maintien de l'allodynie neuropathique. La période de transition entre ces deux phases pourrait varier selon les modèles de neuropathie, conduisant à un décours temporel d'expression du TNFα différent. Cette hypothèse nécessiterait d'être validée par des expériences complémentaires comparant directement différents modèles. De plus, le modèle du manchon se caractérise par une récupération spontanée se produisant entre 12 et 15 semaines après la pose de celui-ci. On peut alors se demander si à ce point temporel les taux de TNFα sont toujours augmentés, ou si là encore il existe une corrélation entre absence de symptômes douloureux et normalisation des taux de TNFα dans les ganglions rachidiens. Il est à noter que l'analyse génomique n'a pas directement détectée d'augmentation du transcrit du TNFα chez les animaux neuropathiques alors qu'une augmentation protéique est visible par Western Blot.

Ce qui pourrait traduire une augmentation de la traduction protéique plutôt qu'une simple augmentation du nombre de transcrits.

D'autres cytokines participent aussi à la physiopathologie de la douleur neuropathique. Par exemple, pendant les heures suivant l'induction d'une neuropathie chez l'animal, une augmentation de la quantité d'IL-1β, cytokine pro-inflammatoire, est rapportée (Lee et al., 2004), ainsi qu'une augmentation d'IL-10, cytokine anti-inflammatoire (Jancalek et al., 2011). Chez l'homme, une augmentation du nombre de transcrits d'IL-10 peut également être observée (Uceyler et al., 2007). Dans un modèle inflammatoire, il a aussi été montré qu'IL-1β est produite par les cellules satellites du ganglion trigeminal (Takeda et al., 2007). Enfin, chez le rat, une compression chronique du nerf sciatique augmente l'expression d'IL-6 dans les ganglions rachidiens (Brazda et al., 2009; Dubovy et al., 2010; Dubovy et al., 2013), et cette augmentation se maintient au moins 4 semaines (Lee et al., 2004). Chez l'homme, la surexpression d'IL-6 semble corrélée à la sévérité de l'allodynie (Ludwig et al., 2008).

Comme pour le TNFα, les expressions et la libération de cytokines semblent dépendre du modèle considéré et du décours temporel étudié. En effet, dans le modèle du manchon, aucune modification à long-terme de l'expression de IL-1β, IL-6 ni IL-10 n'a été retrouvée par qPCR. On peut donc imaginer qu'il existe un recrutement différent des cytokines selon le modèle et la sévérité de la neuropathie. On pourrait également expliquer cela par l'utilisation de techniques de visualisation différentes. En effet, la plupart des études utilisent l'immunohistochimie pour quantifier les taux de cytokines alors que ces méthodes sont peu quantitatives (Takeda et al., 2007; Dubovy et al., 2010). Toutefois, certains auteurs retrouvent ces différences avec des méthodes quantitatives ou semi-quantitative telles que l'enzyme-linked immunosorbent assay (ELISA, *dosage d'immunoabsorption par enzyme liée*), la qPCR ou le Western Blot (Lee et al., 2004; Uceyler et al., 2007; Jancalek et al., 2011; Dubovy et al., 2013). L'hypothèse la plus probable reste le décours temporel étudié. En effet, nous avons étudié l'expression de ces cytokines à plus de 6 semaines, alors que toutes les autres études se placent à des temps proches de la chirurgie, entre 1 et 28 jours (Brazda et al., 2009; Dubovy et al., 2010; Jancalek et al., 2011). On pourrait donc supposer que l'expression d'IL-1β, IL-6 et IL-10 est modulée lors de l'initiation de la neuropathie mais que ces cytokines seraient moins impliquées dans son maintien. Cette hypothèse est étayée par le fait qu'aucune modification d'IL-1β n'est visible dans les ganglions rachidiens à 28 jours post-chirurgie dans le modèle d'une ligature lâche du nerf sciatique (Lee et al., 2004).

3. Impact des traitements sur la neuroinflammation

Dans le modèle de douleur neuropathique périphérique utilisé, nous avons montré que le TNFα, en particulier sa forme membranaire, est augmenté dans le ganglion rachidien en condition neuropathique. De plus, un traitement prolongé par un antidépresseur, tricyclique (Bohren et al., 2013) ou SSNRI (*cf. p.94)*, ou par un β_2-mimétique (Bohren et al., 2013), diminue cette surexpression. Etonnement, un traitement prolongé par la prégabaline conduit également à cet effet anti-TNFα dans les ganglions rachidiens (*cf. p.79)*. Cette action traduit un mécanisme de plasticité puisqu'une dose unique, même importante, de duloxétine ou de prégabaline, n'impacte pas, dans les heures qui suivent, les taux de TNFα dans les ganglions rachidiens (*cf. p.94).* Antidépresseurs et gabapentinoïdes possèdent donc, tous deux, une action anti-neuroimmunitaire lorsqu'ils sont utilisés en traitement prolongé, suggérant l'existence, à un niveau restant à déterminer, d'une convergence d'action de ces deux classes pharmacologiques différentes (Zhu et al., 2008; Wodarski et al., 2009; Bohren et al., 2013; Lee et al., 2013; Kremer et al., 2016).

a. Antidépresseurs et neuroinflammation

Nous avons montré qu'il existait un lien entre les β_2-AR et le TNFα. Mais la question de la signalisation intracellulaire recrutée afin de diminuer la production de cette cytokine reste encore discutée. D'après la littérature, cet effet anti-inflammatoire des β_2-AR, principalement étudié au niveau du système pulmonaire, serait lié à une inhibition d'expression des gènes dépendants de la voie de signalisation NFκB (Ye, 2000; Shore and Moore, 2003). Nous avons observé un résultat similaire dans les ganglions rachidiens dans un contexte de douleur neuropathique : un traitement prolongé par la duloxétine diminue l'expression de gènes de cette voie de signalisation et une inhibition pharmacologique de cette dernière est suffisante pour obtenir un effet antiallodynique (*cf. p.94*). Un des mécanismes permettant d'inhiber cette voie serait d'augmenter le recrutement de son répresseur endogène, IκB-α. En effet, des données *in vitro* obtenues dans les cellules gliales ou les astrocytes montrent que les antidépresseurs tricycliques (Hwang et al., 2008) ou la noradrénaline (Gavrilyuk et al., 2002) augmentent les taux d'IκB-α. Toutefois, ce recrutement n'a pas lieu au niveau transcriptionnel. En effet, nos résultats de

transcriptomique ne rapportent pas de changement apparent de l'expression d'IκB-α. En fait, d'autres groupes montrent que la stimulation des β_2-AR augmente les taux d'AMPc intracellulaires (Farmer and Pugin, 2000; Ye, 2000; Takahashi et al., 2002), conduisant à une stabilisation protéique d'IκB-α (Farmer and Pugin, 2000) qui entraine ainsi une augmentation de sa présence sans que ce mécanisme n'affecte les transcrits.

Un autre mécanisme, recrutant la production de cytokines anti-inflammatoires, pourrait aussi être envisagé. En effet, après une lésion spinale, des administrations répétées de mirtazapine, un antidépresseur tétracyclique, normalisent les taux de TNFα et d'IL-1β et augmentent la production d'IL-10 dans le cerveau (Zhu et al., 2008).

b. Gabapentinoïdes et neuroinflammation

Tout comme les antidépresseurs, les gabapentinoïdes normalisent les taux de TNFα dans les ganglions rachidiens. Mais les voies de signalisation et les mécanismes recrutés pourraient être différents de ceux des antidépresseurs. Une étude récente, conduite dans un modèle de douleur neuropathique, a montré que des injections intrathécales répétées de gabapentine inhibaient la production de cytokines pro-inflammatoires, comme le TNFα, en augmentant l'expression d'IL-10 (Lee et al., 2013). Il est intéressant ici de noter que cette interleukine présenterait un potentiel thérapeutique dans les maladies neuroimmunes (Kwilasz et al., 2015) mais également dans la douleur neuropathique (Ledeboer et al., 2007; Milligan et al., 2012; Song et al., 2016). L'augmentation d'IL-10 par la gabapentine favoriserait aussi l'expression de l'hème oxygénase 1 (HO-1) dans la corne dorsale de la moelle épinière (Bao et al., 2014). HO-1 est une protéine inductible par le stress qui possède des effets anti-inflammatoires et anti-nociceptifs, via notamment la synthèse de monoxyde de carbone (CO), un neurotransmetteur gazeux impliqué dans la modulation des voies nociceptives (Steiner et al., 2001; Rosa et al., 2008; Hervera et al., 2012) et par la diminution d'expression de cytokines pro-inflammatoires (Lee and Chau, 2002; Tai et al., 2009). Une étude a également montré que le CO induisait un rétrocontrôle positif sur la production d'IL-10 et de l'HO-1, amplifiant ainsi ses capacités anti-inflammatoires (Lee and Chau, 2002). Dans les ganglions rachidiens, suite à une lésion nerveuse périphérique, un traitement prolongé par la prégabaline augmente les taux d'IL-10 (Khan et al., 2016), mais aucune donnée n'a pour le moment montré que ce mécanisme passerait par un recrutement de l'HO-1 ou du CO. Il est à

noter que les antidépresseurs facilitent aussi l'expression de l'HO-1, mais via l'activation des voies de signalisation ERK et JNK (Tai et al., 2009; Lin et al., 2012).

Enfin, au site de lésion, une étude rapporte un effet inverse des gabapentinoïdes. En effet, dans les jours suivant la chirurgie, la gabapentine augmenterait les taux de TNFα et d'IL-1β et diminuerait les taux d'IL-10 au niveau du nerf sciatique (Camara et al., 2013). Mais cette étude a été réalisée dans les 5 jours suivant la lésion nerveuse et nécessite confirmation. Elle laisse toutefois supposer que les gabapentinoïdes agiraient différemment sur une neuropathie en développement ou installée.

c. Cibler le TNFα

Afin de manipuler pharmacologiquement le TNFα, les approches les plus utilisées visent à piéger le TNFα plutôt qu'à bloquer ses récepteurs. Des agents anti-TNFα comme l'infliximab, un anticorps monoclonal chimérique humain-souris se liant aux formes solubles et membranaires du TNFα, ou l'etanercept, une protéine de fusion associant le récepteur 2 soluble du TNFα avec une immunoglobuline, sont aujourd'hui utilisés en clinique pour traiter certaines maladies auto-immunes. Chez l'animal, ces médicaments administrés en prévention, ou peu de temps après l'induction d'une neuropathie, possèdent un effet antiallodynique (Goupille et al., 2007; Marchand et al., 2009). Toutefois, l'effet thérapeutique sur une neuropathie déjà installée est moins documenté. Une étude clinique suggère qu'une injection sous-cutanée périspinale de 25 mg d'etanercept soulagerait, chez l'homme, des radiculopathies installées depuis des années (Tobinick and Davoodifar, 2004). Ces résultats n'ont pu être confirmés par une étude utilisant des doses plus faibles (de 0,1 à 1,5 mg), suggérant un effet dose-dépendant (Cohen et al., 2007). De plus, les rapports de cas cliniques sur l'efficacité de ces traitements dans les douleurs neuropathiques se limitent souvent à une seule catégorie de douleurs neuropathiques, les radiculopathies.

Au vu de nos résultats montrant le maintien de taux élevés de TNFα dans les ganglions rachidiens d'animaux neuropathiques, nous avons testé l'effet thérapeutique de ces médicaments sur une neuropathie installée. Nos résultats montrent un potentiel antiallodynique de ces deux médicaments. Néanmoins, les effets biologiques diffèrent partiellement vis-à-vis du mTNFα. L'infliximab, contrairement à l'etanercept, via sa liaison au mTNFα, peut aussi activer une signalisation inverse aboutissant à l'arrêt du cycle cellulaire, à l'apoptose des cellules productrices de TNFα et à une réponse anti-inflammatoire (Horiuchi

et al., 2010). L'infliximab, en plus de neutraliser le TNFα présent, pourrait donc aussi inhiber la production locale de TNFα, amplifiant ainsi l'action bénéfique sur le soulagement de l'allodynie mécanique. De plus, l'affinité de l'infliximab pour le TNFα serait 4 fois supérieure à celle de l'etanercept (Scallon et al., 2002).

Les effets antiallodyniques observés dans notre modèle murin posent la question du potentiel thérapeutique de l'etanercept et de l'infliximab dans le soulagement des douleurs neuropathiques. Néanmoins, les effets indésirables de ces traitements sont nombreux et potentiellement graves, comme le développement de lymphomes (Wong et al., 2012) ou de cancers cutanés non mélanomateux (van Lumig et al., 2015). Mais un des effets indésirables le plus courant est une dépression du système immunitaire pouvant causer l'apparition ou l'aggravation de certaines maladies virales ou bactériennes. Par exemple, il est courant que des patients soignés par traitements anti-TNFα pour de l'arthrite rhumatoïde développent un zona. Par contre, ces patients zostériens ne présentent pas la douleur neuropathique qui accompagne souvent le zona (Javed et al., 2011), suggérant là encore un effet bénéfique des médicaments anti-TNFα sur de telles douleurs. Néanmoins, le ratio bénéfice / risque n'est pas en faveur d'une utilisation des anti-TNFα dans le cadre de douleurs neuropathiques.

II. Douleur neuropathique et système noradrénergique

Il existe deux grands systèmes noradrénergiques. 1) Au niveau périphérique, les neurones post-ganglionnaires sympathiques innervent tous les viscères de l'organisme dont les vaisseaux sanguins et le système immunitaire (Janig, 2014). 2) Au niveau central, les neurones situés dans le tronc cérébral regroupés en 7 noyaux dont le plus important est le groupe A6 (ou locus coeruleus) qui est à l'origine des contrôles descendants noradrénergiques impliqués dans la modulation de la douleur (Pertovaara, 2013).

1. Système noradrénergique périphérique

a. Bourgeonnement sympathique ganglionnaire

Nous observons un bourgeonnement des fibres noradrénergiques dans les ganglions rachidiens, 5 à 8 semaines après la pose d'un manchon autour du nerf sciatique. D'après la littérature, l'origine de ce bourgeonnement dépend du modèle utilisé et de la localisation, proximale (proche des ganglions rachidiens) ou distale (proche des nerfs périphériques), de la lésion (Ramer and Bisby, 1999). En effet, après une lésion proximale, le bourgeonnement sympathique, sous l'influence du facteur de croissance Nerve Growth Factor (NGF, *facteur de croissance neural*) et de la dégénérescence Wallérienne, est issu des fibres non lésées telles que les collatérales sympathiques qui innervent les vaisseaux sanguins ou la dure mère. Par contre, après une lésion distale du nerf sciatique, le bourgeonnement, indépendant du NGF, est produit par les fibres sympathiques distales lésées qui régénèrent. Dans le modèle de compression du nerf sciatique utilisé, le manchon est placé autour du tronc commun du nerf sciatique, soit une lésion plutôt proximale. Le bourgeonnement proviendrait alors des vaisseaux sanguins situés à proximité des ganglions rachidiens et de la dure mère plutôt que d'axones sympathiques sectionnés. Une étude récente, utilisant ce modèle du manchon et un modèle de section des branches terminales du nerf sciatique, chez le rat, n'observe pas ce phénomène de bourgeonnement, ni à 2 semaines post-chirurgie, ni à 6 semaines (Nascimento et al., 2015). Bien que nos techniques expérimentales semblent proches, le faible nombre d'animaux qu'ils ont utilisé, le nombre de sections analysées et le fait qu'ils se soient focalisés sur le seul ganglion rachidien L4, sans regarder L5 ni L6 pourrait expliquer cette absence de détection. En effet, ce bourgeonnement au sein du

ganglion rachidien peut présenter des différences anatomiques et ne couvre pas l'ensemble du ganglion rachidien (observations personnelles). De plus, ces auteurs n'ont comptabilisé que les fibres au contact des corps cellulaires des neurones, or il existe une transmission volumique importante pour les fibres noradrénergique qui pourrait résulter d'un bourgeonnement plus périphérique.

Ce bourgeonnement serait un substrat de l'action des antidépresseurs. Ces derniers agiraient en bloquant la recapture de la noradrénaline par les fibres sympathiques, augmentant ainsi les taux extracellulaires de noradrénaline, qui va ensuite agir sur les β_2-AR des cellules satellites. Ce bourgeonnement ne débute en général qu'une à deux semaines après une lésion nerveuse (Ramer and Bisby, 1998). Dans nos travaux, nous commençons toujours les traitements, qu'ils soient aigus ou prolongés, après une période postopératoire d'au minimum 15 jours, c'est-à-dire pendant le développement de ce bourgeonnement. Le délai thérapeutique des antidépresseurs pourrait ainsi s'expliquer par le fait que la mise en place des fibres noradrénargiques dans les ganglions rachidiens est encore incomplète à cette période postopératoire. Il faudrait confirmer cette théorie en effectuant une comparaison plus précise du décours temporel thérapeutique et de la progression du bourgeonnement sympathique à divers moments du développement de la neuropathie. Il est à noter que les antidépresseurs sont inefficaces dans notre modèle lorsqu'ils sont administrés pendant les deux premières semaines post-chirurgie (Salvat et al., soumis).

Il serait alors intéressant d'explorer l'existence de ce bourgeonnement dans d'autres étiologies de la douleur neuropathique, là où les antidépresseurs sont moins efficaces. Par exemple, en clinique comme chez l'animal, les antidépresseurs ont peu ou pas d'effet thérapeutique suite à une lésion médullaire (Hama and Sagen, 2007; Cardenas and Felix, 2009). De même, lorsque l'on ligature la branche principale de la division mandibulaire afin de mimer une névralgie chez le rat, aucun bourgeonnement n'est observé dans le ganglion du trijumeau correspondant (Bongenhielm et al., 1999). Ceci pourrait s'expliquer par le fait que les afférences sympathiques sont moins nombreuses dans cette région qu'autour des nerfs spinaux (Hoffmann and Matthews, 1990). Comme nous l'avons décrit, ce phénomène de bourgeonnement peut être sous la dépendance du NGF. Ce facteur de croissance est synthétisé par les neurones sensoriels ou les cellules satellites gliales après une lésion nerveuse périphérique (Shinder et al., 1999; Zhou et al., 1999). On pourrait alors poser l'hypothèse d'une différence de production de NGF entre le ganglion du trijumeau et le

ganglion rachidien, avec peu ou pas d'expression de NGF dans le cas de la névralgie. Ceci est étayé par le fait que lorsque l'on surexprime ce facteur de croissance chez un animal naïf, on voit apparaitre un bourgeonnement sympathique dans le ganglion du trijumeau (Davis et al., 1996; Walsh and Kawaja, 1998). Chez le patient, la névralgie est peu sensible à l'effet des antidépresseurs, le traitement de première intention de ces douleurs étant la carabamazépine, un antiépileptique (Campbell et al., 1966; Fields, 1996; Cruccu et al., 2016). Enfin, des résultats préliminaires de notre équipe, montrent la présence de ce bourgeonnement dans les ganglions rachidiens de souris obèses diabétiques, développant une allodynie mécanique sensible à la nortryptyline. Il semblerait alors que l'efficacité du mécanisme « chronique » des antidépresseurs (i.e. leurs effets anti-neuroinflammatoires) soit corrélée à la présence du bourgeonnement sympathique périphérique, présence dépendante de l'étiologie de la douleur neuropathique considérée.

b. Les catécholamines circulantes

Une autre source adrénergique périphérique est fournie par les glandes surrénales. Aucune étude n'a vérifié si, suite à une lésion nerveuse périphérique, une augmentation des taux de catécholamines produites par les glandes surrénales existait. De plus, les effets des antidépresseurs sur la recapture des monoamines à ce niveau sont peu connus bien que la médullosurrénale possèdent des transporteurs de recapture de la noradrénaline (Kippenberger et al., 1999). Par contre, un effet antinociceptif d'une greffe de cellules chromaffines de glandes surrénales au niveau de l'espace subarachnoïdien spinal après une lésion nerveuse a été observé (Ginzburg and Seltzer, 1990; Hama and Sagen, 1993; Brewer and Yezierski, 1998; Siegan and Sagen, 1998), suggérant un effet bénéfique d'un apport de noradrénaline au niveau spinal.

c. Les adrénocepteurs mis en jeu : β_2-AR

Nos résultats ont montré que la noradrénaline endogène, recrutée par les antidépresseurs, peut interagir via les β_2-AR des ganglions rachidiens, situés sur les cellules satellites, et que la stimulation directe des β_2-AR par des agonistes permet de réduire l'allodynie mécanique. Ces résultats sont cohérents avec les effets du système sympathique et de la noradrénaline sur le système immunitaire. En effet, la noradrénaline via l'activation des β_2-AR, inhibe l'immunité innée et acquise, diminuant ainsi la production et la libération de cytokines pro-

inflammatoires comme le TNFα et l'IL-1β, et augmentant la production de l'IL-10 anti-inflammatoire (Elenkov et al., 2000; Bellinger and Lorton, 2014). En outre, la myélopoïèse, c'est-à-dire la production des monocytes et des macrophages, est également sous le contrôle négatif des β_2-AR. Au contraire, la lymphopoïèse, plus particulièrement la production des lymphocytes T, est, elle, sous contrôle positif des β_2-AR (Elenkov et al., 2000; Bellinger and Lorton, 2014).

2. Le système noradrénergique central

a. Les voies noradrénergiques descendantes inhibitrices

Les résultats que nous avons obtenus après administration d'une dose forte, unique, de duloxétine suggèrent une action centrale de la noradrénaline dans un contexte de douleur neuropathique (*cf. p.94)*. En effet, suite à une lésion nerveuse périphérique, on peut parfois observer un bourgeonnement des fibres descendantes noradrénergiques dans la moelle épinière, ainsi qu'une augmentation de la libération de la noradrénaline (Ma and Eisenach, 2003; Hayashida et al., 2008). Dans le locus cœruleus, on observe également des modifications d'expression de la tyrosine hydroxylase (TH) et des transporteurs de la noradrénaline (Alba-Delgado et al., 2013; Llorca-Torralba et al., 2016). Si l'activité spontanée de ce noyau n'est pas altérée lors de l'initiation d'une douleur neuropathique (Viisanen and Pertovaara, 2007; Alba-Delgado et al., 2013; Bravo et al., 2014; Llorca-Torralba et al., 2016), certains paramètres de son activité sont affectés à plus long terme (Alba-Delgado et al., 2013; Llorca-Torralba et al., 2016), preuve que le système noradrénergique central se modifie suite à l'induction d'une douleur neuropathique.

Comme les antidépresseurs inhibent la recapture des monoamines, leur effet thérapeutique pourrait passer par une action directe sur les contrôles descendants inhibiteurs noradrénergiques (*cf. p.94*). De plus, les antidépresseurs pourraient également agir au niveau supraspinal, en influençant les composantes somatosensorielle et affective de la douleur neuropathique (Mico et al., 2006). Ils permettent, par exemple, de restaurer les paramètres de l'activité du locus cœruleus altérés par la douleur neuropathique (Alba-Delgado et al., 2012; Llorca-Torralba et al., 2016).

b. Les adrénocepteurs mis en jeu : α-AR

Nous avons montré que la duloxétine, administrée en aigüe, peut recruter la noradrénaline via l'activation des voies descendantes noradrénergiques inhibitrices, et que cette dernière agirait ensuite via α_{2A}-AR et non α_{2C}-AR afin de soulager, transitoirement, la douleur neuropathique. Il est intéressant de noter qu'après une lésion nerveuse périphérique, il existe une augmentation d'expression des α_2-AR, et principalement du sous-type α_{2A} dans la moelle épinière (Giroux et al., 1999; Pertovaara, 2013), ainsi qu'au niveau supraspinal dans le locus cœruleus (Alba-Delgado et al., 2013; Llorca-Torralba et al., 2016). En accord avec nos résultats, il a été montré que l'utilisation d'agonistes des α_2-AR réduit la douleur neuropathique alors que des agonistes des α_1-AR seraient pro-nociceptifs (Pertovaara, 2013). En outre, comme pour les β_2-AR, les α-AR interviennent également dans la régulation du système immunitaire. Les $\alpha_{1/2}$-AR régulent positivement la production des lymphocytes B, alors qu'ils régulent négativement la production des neutrophiles (Bellinger and Lorton, 2014).

Remarque : Au niveau périphérique (fibres sympathiques) comme au niveau central (fibres descendantes noradrénergiques), les α_2-AR présents sur les terminaisons noradrénergiques (au niveau pré-synaptique) contrôlent la libération de noradrénaline (Elenkov et al., 2000).

III. Recherche translationnelle

1. Modèle animal de douleur neuropathique

a. Validité étiologique

Les conditions conduisant à la pathologie dans le modèle animal doivent se rapprocher au mieux de celles connues chez l'homme. Les étiologies possibles des douleurs neuropathiques sont assez bien identifiées, contrairement à d'autres pathologies du système nerveux. Ces douleurs sont causées par des lésions nerveuses traumatiques ou chirurgicales, des troubles métaboliques (diabète, éthylisme) ou encore l'action neurotoxique iatrogène de médicaments ou des infections virales (VIH). La pose d'un manchon en polyéthylène ne reproduit pas une réalité clinique, mais en mime certains aspects. En effet, cette pose comprime le nerf sciatique (Mosconi and Kruger, 1996; Benbouzid et al., 2008c; Yalcin et al., 2014), une des étiologies possibles de douleurs neuropathiques, comme par exemple les compressions nerveuses chroniques par hernie discale ou par le muscle piriforme.

b. Validité symptomatique

Les symptômes observés chez l'animal doivent se rapprocher de ceux observés en clinique. L'hyperalgésie thermique et l'allodynie mécanique et thermique sont rapportées par les patients souffrant de douleurs neuropathiques périphériques (Attal et al., 2008). Le modèle de compression du nerf sciatique entraine bien une allodynie mécanique statique persistante dans le temps, mais il n'entraine qu'une hyperalgésie thermique au chaud transitoire et les essais pour détecter une allodynie au froid se sont avérés techniquement peu probants, bien que ce symptôme s'observe dans la plupart des modèles (Gregory et al., 2013). De plus, chez le patient, les symptômes d'allodynie sont plus couramment d'ordre dynamique que statique (Hansson, 2003). Chez l'animal, l'allodynie mécanique statique est évaluée grâce aux filaments de von Frey. Malgré l'utilisation de protocoles parfois différents entre laboratoires, ce test permet d'avoir des valeurs stables et reproductibles (Barrot, 2012).

Dans certaines de nos expériences, nous utilisons à la fois des souris C57BL/6J femelles et mâles. La sensibilité mécanique statique est différente entre les deux sexes, les

femelles possédant un seuil plus bas que les mâles, mais cette différence s'avère présente et stable à la fois en conditions physiologique et neuropathique.

Depuis quelques années, des laboratoires essaient de développer, chez le rongeur, un test pour mesurer l'allodynie mécanique dynamique : le test du pinceau souple (paint-brush test). Plusieurs protocoles existent. Certains codifient la réponse de l'animal selon 3 critères suite à la caresse de la surface plantaire par un pinceau (Sasaki et al., 2008; Yamamoto et al., 2016). D'autres font la moyenne du nombre de retraits de la patte sur 5 applications de 5 secondes (Thibault et al., 2008). Enfin, Bourane et al. (2015) ont mis en place un protocole reproductible, associant les deux précédents ; chaque test, répété 3 fois, comprend 3 épisodes de stimulation par le pinceau effectués à 10 secondes d'intervalle et une grille d'évaluation du comportement (Bourane et al., 2015). Nous n'avons pas encore réalisé ce test dans notre modèle mais c'est une prochaine étape indispensable que nous souhaitons mettre en place.

Il apparaît aujourd'hui nécessaire de rechercher des paramètres, peut-être indirects, mettant en évidence chez l'animal la douleur plus que la nociception. Les douleurs spontanées sont en effet couramment rapportées en clinique, mais leur évaluation repose essentiellement sur la verbalisation, ce qui la rend difficile chez l'animal. Des tests de conditionnement de place ou d'évitement actif permettent de révéler, indirectement, la présence de la composante aversive de la douleur spontanée (Baastrup et al., 2011; King et al., 2011; Qu et al., 2011; Bravo et al., 2012). Toutefois, ces tests restent techniquement difficiles à réaliser et nécessitent un grand nombre d'animaux.

Environ 30% des patients souffrant de douleurs neuropathiques développent des troubles de l'humeur et l'utilisation de tests comportementaux dans le modèle que nous utilisons permet également de mettre en évidence l'apparition de phénotypes de type anxieux et dépressifs (Yalcin et al., 2011; Barthas et al., 2015).

c. Validité thérapeutique

Le modèle animal doit présenter un profil pharmacologique de réponse aux traitements semblable à celui observé chez l'homme. Le modèle utilisé répond aux traitements existants, comme les antidépresseurs et les gabapentinoïdes, à des doses moyennes lors d'un traitement prolongé. De plus, le délai thérapeutique est similaire à celui observé en clinique (Attal et al., 2010; Sharma et al., 2010) renforçant ainsi la pertinence du modèle utilisé. Cet

effet s'observe pour les différents antidépresseurs efficaces en clinique, tels que les antidépresseurs tricycliques (amitriptyline, nortriptyline, désipramine), les SSNRI (venlafaxine, duloxétine, milnacipran), ainsi que pour la prégabaline et la gabapentine (*cf. p.79*; *p.94*; Benbouzid et al., 2008a; Yalcin et al., 2009b). Par contre, comme observé chez l'homme, la fluoxétine, inhibiteur sélectif de la recapture de la sérotonine, est inefficace dans ce modèle (Benbouzid et al., 2008a). En clinique, les douleurs neuropathiques sont insensibles aux anti-inflammatoires non stéroïdiens et cela a pu être confirmé dans ce modèle avec le kétroprofène (Benbouzid et al., 2008c). Enfin, même si les opiacés peuvent montrer une efficacité, leur utilisation n'est pas recommandée en première intention chez l'homme (Finnerup et al., 2015), principalement en raison des problèmes de tolérance et des effets indésirables. Dans ce modèle animal, la morphine est efficace en aigüe, à fortes doses, mais cet effet montre une tolérance rapide (Benbouzid et al., 2008c).

2. Utiliser un modèle animal pour prédire l'efficacité et comprendre le mécanisme d'action des traitements de la douleur neuropathique

La recherche translationnelle est aujourd'hui nécessaire afin d'améliorer la prise en charge thérapeutique de la douleur neuropathique, en transposant rapidement les avancées préclinique à la clinique. Nous avons effectué une recherche translationnelle en comparant les taux plasmatiques de duloxétine entre l'homme et la souris afin de déterminer quel mécanisme était mis en jeu en clinique, de telles comparaisons nécessitent certaines règles.

a. Biais expérimentaux et solutions proposées

Beaucoup d'auteurs critiquent la présence de biais expérimentaux, ne reflétant pas la clinique, dans les expériences précliniques (pour revue : Rice et al., 2008; Berge, 2011). Par exemple, les médicaments utilisés contre la douleur neuropathique sont souvent testés à dose fixe chez l'animal, en une seule administration, alors qu'en clinique les doses peuvent être progressivement augmentées pour des raisons de tolérance et dans le but de limiter les effets indésirables, et une exposition au long cours est souvent requise pour une efficacité optimale. En fait, le métabolisme et la distribution tissulaire d'un médicament peuvent être significativement différentes selon qu'il soit administré une seule fois ou au long cours lors d'un traitement prolongé. Nous pouvons ainsi prendre l'exemple de la gabapentine pour

laquelle son absorption du tractus gastro-intestinal vers le sang, ainsi que du sang vers le liquide céphalorachidien, est saturable (Stewart et al., 1993; Luer et al., 1999). Après une administration unique de gabapentine, seule une faible quantité atteint le système nerveux central, son principal site d'action (Welty et al., 1993; Berge, 2011). De plus, même avec la plus petite dose, aigüe, efficace chez l'animal, les concentrations plasmatiques mesurées sont 3 à 15 fois supérieures à celles mesurées chez l'homme dans des conditions thérapeutiques classiques (Whiteside et al., 2008). Bien que cette différence puisse paraître modérée, ces fortes concentrations peuvent, dans le cas de la gabapentine, recruter un mécanisme anti-nociceptif périphérique, peu ou pas présent chez le patient, ce qui peut également expliquer le fait que ce composé soit plus efficace dans le modèle animal qu'en clinique (NNT = 6,3) (Berge, 2011; Finnerup et al., 2015). C'est également ce que nous observons à travers nos différents résultats. Les concentrations plasmatiques de duloxétine chez le patient sont proches de celles observées chez l'animal sous traitement prolongé à faibles doses ; alors que sous traitement aigu, à fortes doses, les concentrations plasmatiques mesurées chez l'animal sont 12 fois plus élevées qu'en clinique. Dans ce cas, il existe un recrutement du système anti-nociceptif central chez la souris, recrutement peu probable chez l'homme. De même nous avons montré qu'une administration unique, intrapéritonéale, de prégabaline avait une action antiallodynique transitoire, probablement via un mécanisme différent de celui recruté par un traitement prolongé (*cf. p.31*).

Un autre biais existe toutefois dans notre étude, l'administration orale chez l'animal est assez étalée dans le temps, bien que la prise hydrique journalière s'effectue sur un temps rapproché, en début de phase d'activité, chez le rongeur. Nous n'avons donc pas réussi à reproduire les variations plasmatiques décrites chez le patient (entre le pic et le taux résiduel).

Pour réduire le nombre de biais expérimentaux, un consortium de recherche préclinique a proposé une liste de différents paramètres à considérer et à discuter dans toute recherche préclinique à caractère translationnel (Rice et al., 2008). Parmi ces points, la pharmacocinétique occupe un paragraphe, dans lequel les auteurs encouragent à faire le lien entre les concentrations plasmatiques mesurées chez l'animal et les données existantes en clinique ; ce que nous avons réalisé dans notre étude (*cf. p.94*). Cette préoccupation est peu présente dans la littérature et les publications précliniques s'intéressent rarement aux données de pharmacocinétique, aux taux plasmatiques ni aux taux d'exposition des tissus à

un médicament (Whiteside et al., 2008; Berge, 2011). Toutefois quelques rares articles ont essayé de mettre en parallèle la pharmacologie d'antalgiques chez l'homme et chez l'animal (Fishbain et al., 2000; Pullar and Palmer, 2003; Blackburn-Munro and Erichsen, 2005).

b. Métabolisme de la duloxétine chez l'homme et la souris

Chez l'homme, la duloxétine est métabolisée par deux cytochromes, P450-2D6 et 1A2, en deux métabolites principaux, inactifs, le glucuroconjugué 4-hydroxyduloxétine et le sulfoconjugué 5-hydroxy-6-méthoxyduloxétine (Lantz et al., 2003; Rodieux et al., 2015). Les cytochromes P450 constituent une superfamille de 57 gènes codant des enzymes qui métabolisent un grand nombre de médicaments (Gueguen et al., 2006). Chez la souris (*Mus musculus*) le cytochrome P450-1A2 est bien présent dans son génome, par contre le cytochrome P450-2D6 n'existe pas, son équivalent est le cytochrome P450-2D22 (Blume et al., 2000). Grâce à la base de données Ensembl (http://www.ensembl.org/index.html), on obtient une comparaison de gènes entre espèces. Les gènes CYP2D6 codant le cytochrome P450-2D6 chez l'homme (*Homo sapiens*) et *Cyp2d22* codant l'équivalent chez la souris (*Mus musculus*) sont des gènes orthologues. Ils possèdent 75% de similarités. Les gènes CYP1A2 et *Cyp1a2* codant le cytochrome P450-1A2 chez l'homme et la souris présentent 73% de similitudes. Les gènes et les cytochromes qui en découlent étant donc fortement conservés entre l'homme et la souris et les métabolites de la duloxétine étant inactifs, on peut donc considérer qu'il existe une homologie de métabolisme entre l'homme et la souris.

Dans le cas d'un patient souffrant de douleurs neuropathiques traité par la duloxétine, nos résultats montrent une réduction de la demi-vie de cette dernière, aux alentours de 6 heures (contre en moyenne 12 heures chez le sujet sain). Plusieurs explications peuvent être évoquées. L'activité des cytochromes impliqués, et donc la biodisponibilité de la duloxétine, pourrait être affectée par des interactions médicamenteuses (Gueguen et al., 2006). Mais nous avons, dans notre étude translationnelle, choisi comme critère d'exclusion les patients co-traités avec des substrats de ces deux cytochromes, tels que la fluvoxamine (P450-1A2) ou l'oméprazole (P450-2D6). Les analyses pharmacocinétiques de population ont aussi montré que les concentrations plasmatiques de duloxétine étaient réduites de moitié chez les fumeurs comparativement aux non fumeurs (Knadler et al., 2011). Nous n'avons pas exclu les fumeurs, à cause du faible taux de recrutement de notre étude, mais actuellement toutes les informations recueillies le

sont sur des patients non fumeurs. Il a également été montré que l'activité de ces cytochromes pouvait varier avec l'âge et le sexe. L'ensemble de ces facteurs pourrait expliquer une variabilité interindividuelle concernant l'efficacité thérapeutique et la tolérance de la duloxétine. Toutefois, nos résultats montrent une très faible variabilité interindividuelle (seulement 6%, que l'on se place au pic plasmatique ou au taux résiduel) sans implication du sexe. Tous ces paramètres n'expliquent donc pas la réduction de la demi-vie de la duloxétine observée chez nos patients. On pourrait alors supposer l'existence d'une adaptation, au niveau transcriptionnel ou catalytique, de ces cytochromes par la prise de cet antidépresseur sur des périodes très longues.

Les nombreux modèles animaux de douleur neuropathique constituent une boîte à outils nécessaire et importante pour décrypter les mécanismes physiopathologiques, prédire l'efficacité des traitements et comprendre leur mécanisme d'action. A travers notre étude sur la duloxétine, nous avons essayé de mettre l'accent sur la pharmacocinétique de cette molécule chez l'homme et l'animal afin de mieux comprendre le mécanisme analgésique observé en clinique. Notre étude se démarque par le progrès qu'elle apporte de par sa conception résolument tournée vers la clinique.

PERSPECTIVES

Dans cette étude mécanistique des traitements de la douleur neuropathique dans un modèle murin, nous avons recherché des acteurs moléculaires communs entre différents traitements principalement les antidépresseurs, les gabapentinoïdes et les β_2-mimétiques. Nous concluons que, bien qu'agissant sur des cibles et des voies de signalisation différentes, ces traitements conduisent à un effet anti-neuroinflammatoire dans les ganglions rachidiens. Dans ces derniers, nous avons également mis en évidence des modifications de la matrice extracellulaire lors d'une lésion nerveuse périphérique, atténuées par un traitement prolongé par la duloxétine. A terme, le décours temporel de ces manifestations cellulaires et moléculaires serait sans doute important à explorer.

Comme nous l'avons plusieurs fois suggéré, la douleur neuropathique entrainerait une cascade de modifications neuroinflammatoires débutant à la périphérie, au site de lésion puis dans les ganglions rachidiens, avant d'atteindre le système nerveux central, au niveau spinal et supraspinal. Il serait alors intéressant d'explorer ce qui se passe dans la corne dorsale de la moelle épinière après un traitement prolongé par les antidépresseurs ou les anticonvulsivants. Est-ce que les modifications cellulaires et moléculaires localisées dans le ganglion rachidien, entraînent, en cascade, des modifications de l'activation microgliale via une diminution de MCP-1 puis une restauration du potentiel d'équilibre anionique diminuant ainsi l'hyperexcitabilité des neurones ? Si c'est le cas, le décours temporel de ces phénomènes sera également à étudier.

Concernant les acteurs communs entre les antidépresseurs et les β_2-mimétiques, nous avons montré que ces deux traitements nécessitaient la présence à la fois des β_2-AR et des récepteurs DOP. La question est alors de savoir quel est le lien mécanistique entre ces deux récepteurs. Est-ce que ces récepteurs font parti d'une même cascade thérapeutique, avec les récepteurs DOP en aval des β_2-AR, ou bien font-ils parti de deux cascades complémentaires ? Il serait également intéressant de connaître les types cellulaires exprimant ces récepteurs, ainsi que ceux produisant les peptides opioïdes endogènes, pour retracer une histoire mécanistique complète de l'action des antidépresseurs et des β_2-mimétiques dans les ganglions rachidiens.

Enfin, nous avons montré que la noradrénaline en provenance du système sympathique périphérique était la source indispensable au traitement prolongé par les antidépresseurs, plaçant ainsi le bourgeonnement sympathique au centre de l'efficacité thérapeutique de ces molécules. Il serait alors intéressant de vérifier la présence de ce

bourgeonnement dans différents modèles de douleurs neuropathiques traumatiques, mais également métaboliques ou iatrogènes, afin de corréler l'efficacité de ces molécules à la présence de ce phénomène.

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Mélanie KREMER

Analyse mécanistique des traitements de la douleur neuropathique

Résumé

La douleur neuropathique est due à une lésion ou une pathologie du système nerveux somatosensoriel. La prégabaline, un anticonvulsivant, et la duloxétine, un antidépresseur, sont des traitements de référence, efficaces chez un tiers des patients. Mieux comprendre leurs mécanismes d'action est crucial pour améliorer leur tolérance et leur efficacité. En utilisant un modèle murin de douleur neuropathique périphérique, nous montrons que : 1) la prégabaline, dont l'action est indépendante du système opioïdergique, agit sur la composante neuroimmunitaire périphérique de la douleur ; 2) la duloxétine agit via deux mécanismes indépendants, l'un central (contrôles descendants) pour un traitement aigu et l'autre périphérique (ganglion rachidien) pour un traitement chronique. Dans ce cas, l'analyse transcriptomique met en évidence une inhibition de l'inflammation neurogène. La comparaison des taux plasmatiques de duloxétine chez l'homme et chez la souris suggère une action périphérique chez l'homme.

Mots-clés : douleur neuropathique, duloxétine, prégabaline, récepteur δ des opioïdes, récepteur β_2-adrénergique, TNFα, ganglion rachidien

Abstract

Neuropathic pain is caused by a lesion or a disease of the somatosensory nervous system. Pregabalin, an anticonvulsant, and duloxetine, an antidepressant, are the standard treatments, effective in one-third of patients. A better understanding of their mechanisms of action is a crucial point to improve their tolerance and efficiency. By using a murine model of peripheral neuropathy, we have shown that : 1) pregabalin, whose effect is independent from the opioid system, acts on the peripheral neuroimmune component of pain ; 2) duloxetine acts via two independent mechanisms, one central (descending controls) for an acute treatment and the other peripheral (dorsal root ganglia) for a chronic treatment. In this case, transcriptomic analysis hightlights an inhibition of the neurogenic inflammation. Comparison of duloxetine plasmatic levels in humans and mice suggests a peripheral action in humans.

Keywords: neuropathic pain, duloxetine, pregabalin, δ opioid receptor, β_2-adrenoceptor, TNFα, dorsal root ganglia