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Role of spinal serotonin 5-HT2A receptor in 2′,3′-dideoxycytidine-induced neuropathic pain in the rat and the mouse

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Abstract

Several lines of evidence suggest that descending serotoninergic facilitatory pathways are involved in neuropathic pain. These pathways may involve 5-HT2A receptors known to play a role in spinal and peripheral sensitization. The implication of this receptor in neuropathy was investigated in a model of peripheral neuropathy induced by 2′,3′-dideoxycytidine, a nucleoside analogue with reverse transcriptase inhibitory properties used in HIV/AIDS therapy. Four days after a single 100 mg/kg i.v. administration in the tail vein, mitochondrial alterations in nociceptive and non-nociceptive dorsal root ganglion cells were observed at the lumbar level. These alterations were not associated with TUNEL labelling or with modification of the total number of dorsal root ganglion cells. At the same time point, 5-HT2A receptor immunolabelling was increased throughout the dorsal horn (by 49.5% in layer II and 57.8% in layer III). The number of 5-HT2A receptor immunoreactive neurons in the dorsal root ganglion was also increased by 30.7%. Four days after 2′,3′-dideoxycytidine administration, rats had developed thermal allodynia as well as mechanical hyperalgesia and allodynia, which dose-dependently decreased after epidural injection of MDL 11,939, a 5-HT2A receptor antagonist. Moreover, 5-HT2A receptor knock-out mice did not develop 2′,3′-dideoxycytidine-induced neuropathy whereas their control littermates displayed a neuropathy comparable to that observed in rats. Our data show that 2′,3′-dideoxycytidine-induced neuropathy is associated with alterations of nociceptive and non-nociceptive peripheral cells and that the 5-HT2A receptor is involved in the peripheral sensitization of nociceptors as well as in a wide central sensitization of dorsal horn neurons.

Keywords: 5-HT2A receptor; Anti-retroviral treatment; Immunocytochemistry; Knock-out mice; Pain behaviour; Ultrastructure; Zalcitabine

1. Introduction

Synthetic 2′,3′-dideoxycytidine (ddC) is a nucleoside analogue with reverse transcriptase inhibitory (NRTI) properties that is used for the treatment of patients infected by the human immunodeficiency virus (HIV). Since the introduction of this therapeutic drug family, the incidence of painful peripheral neuropathy has significantly increased (Dubinsky et al., 1989; Berger et al., 1993; Cherry et al., 2003). The main cellular effect of NRTI that may be at the origin of peripheral neuropathy is a mitochondrial toxicity. Indeed, a high

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incidence of abnormal mitochondria in peripheral axons accompanied by a depletion of mitochondrial DNA was found in patients with ddC-neuropathy (Dalakas et al., 2001). Recently, a model of ddC-induced peripheral neuropathy characterized by mechanical hypersensitivity and allodynia has been developed in the rat (Joseph and Levine, 2004). Caspase inhibitors (Joseph and Levine, 2004), inhibitors of mitochondrial electron transport (Joseph and Levine, 2006) and intracellular calcium modulators (Joseph et al., 2004) were reported to attenuate ddC-induced neuropathy.

In humans, neuropathic pain syndromes, including those induced by NRTI, are difficult to manage (Sindrup and Jensen, 1999; Woolf and Mannion, 1999). Tricyclic antidepressants, one of the main treatments of these pains (Sindrup and Jensen, 1999), have an analgesic effect that may result from both supraspinal and spinal actions (Mico et al., 2006). However, in animals, the development and maintenance of chronic pain states seem to be dependent on descending 5-HT facilitatory pathways (Urban and Gebhart, 1999; Pertovaara, 2000; Porreca et al., 2002; Ren and Dubner, 2002; Vanegas and Schaible, 2004) involving at least the 5-HT3 receptor (Suzuki et al., 2004). Furthermore, 5-HT2A receptor (5-HT2AR) that is strongly expressed by primary sensory neurons and spinal neurons (Doly et al., 2004) is involved in the sensitization of both peripheral (Grubb et al., 1988; Rueff and Dray, 1992) and spinal (Hori et al., 1996; Li et al., 1999) nociceptive cells. 5-HT2AR are also involved in pain at the peripheral level in numerous inflammatory states in which they are responsible for 5-HT induced hyperalgesia (Abbott et al., 1996; Doi-Saika et al., 1997; Tokunaga et al., 1998; Okamoto et al., 2002; Kjorsvik Bertelsen et al., 2003; Wei et al., 2005; Kayser et al., 2007). Furthermore, inflammation is accompanied by a strong up-regulation of 5-HT2AR expression in dorsal root ganglia (DRG) (Wu et al., 2001; Liu et al., 2005).

To validate the model of ddC-induced neuropathy in the adult rat, we firstly looked for morphological alterations of DRG neurons to determine whether mitochondrial alterations and/or apoptosis could support the appearance of hyperalgesia and allodynia evidenced by appropriate behavioural tests. In parallel, the implication of the 5-HT2AR in ddC-induced neuropathy at the spinal and peripheral levels was indirectly assessed by studying its expression in DRG and spinal dorsal horn by immunocytochemistry. We also evaluated the effect of an epidural injection of a 5-HT2AR antagonist on the appearance of mechanical allodynia and hyperalgesia in ddC-treated rats. Finally, we investigated the effect of the absence of the 5-HT2AR, in knock-out 5-HT2AR−/− mice, on ddC-induced pain behaviour.

2. Materials and methods

2.1. Animals

Male Wistar rats (CER Janvier, Le Genest St Isle, France) weighing 180–220 g, 5-HT2AR−/− mutant mice (Fiorica-Howells et al., 2002) and their paired wild-type mice were used in these studies. Genotyping was carried out by polymerase chain reaction on genomic DNA from tail biopsies using appropriate primers (Fiorica-Howells et al., 2002). Wild-type and 5-HT2AR−/− mice were the product of mating between heterozygous couples raised on 129 Sv/Ev/Taq genetic background. In some cases, heterozygous mice were also used in the experiments. Males and females were separated at weaning, and only male mice were used, at 2–3 months of age. Rats and mice were kept under a regular 12-h day/night cycle in a temperature and humidity controlled environment with free access to food and water. Experiments were done in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and following the guidelines published in a Guest Editorial in *Pain* on ethical standards for investigation of experimental pain in animals (Committee for Research and Ethical issues of the I.A.S.P., 1980). Experimental conditions also matched the official requirements of the French ''Ministère de l'Agriculture et de la Pêche'', permission number to MC: 75-819.

2.2. Drugs administration

Rats received a single i.v. injection, in the tail vein, of 100 mg/kg ddC (Sigma, Saint Quentin Fallavier, France) in 0.9% NaCl at a final dilution of 100 mg/ml, under 60 mg/kg pentobarbital (Ceva Santé Animale, Libourne, France) intraperitoneal (i.p.) anaesthesia. This dose was chosen because lower doses of ddC (25–50 mg/kg), corresponding to those used by Joseph et al. (2004), did not induce any significant clinical symptoms in our model. This discrepancy may be accounted for by the difference in rat strain, since Joseph et al. used Sprague–Dawley rats while we used Wistar rats. A group of control rats received the same volume of the vehicle (NaCl 0.9%). In order to determine the dose and route of administration of ddC necessary to obtain a strong neuropathy (characterized by mechanical allodynia and hyperalgesia), preliminary experiments were conducted on sv129 mice (CER Janvier, Le Genest St Isle, France) which have a genetic background similar to that of 5-HT2AR−/− mutants (Fiorica-Howells et al., 2002). Since intravenous injections were difficult to perform in mice, the drug was administered i.p. Two 500 mg/kg ddC i.p. injections were given at four days interval. 5-HT2AR−/− ($n = 5$), 5-HT2AR+/+ ($n = 7$) and 5-HT2AR+/− ($n = 14$) mice were injected in parallel. MDL 11,939 (Tocris, Ellisville, Missouri, USA), a specific 5-HT2AR antagonist (Dudley et al., 1988), was prepared at 10 mM in saline acidified with acetic acid (final pH 6.5). Epidural injection of MDL 11,939 was performed directly through the intact skin of rats between the L5 and L6 vertebrae. Using a Hamilton syringe with a 26-gauge needle, MDL 11,939 was given in a volume of 25 μl at a concentration range of 1–100 μM from the 10 mM solution diluted in artificial cerebro-spinal fluid (aCSF, Harvard Apparatus, Les Ullis, France). An equivalent

number of control rats received the same volume of drug-free aCSF. Both groups of rats were injected under transient volatile anaesthesia with 33% O_2, 67% N_2O and 2% isoflurane (Aerrane, Baxter, Maurepas, France), and delivered via a tight-fitting mask.

2.3. Behavioural tests

Behavioural experiments were done between 10.00 A.M. and 16.00 P.M. in a quiet temperature-controlled room reserved for the tests to avoid variations linked to the environment. In addition, to minimize the stress, animals spent at least three days in the animal facility before the beginning of the experiments. Animals were habituated to the behavioural testing apparatus and environment for 30 min before each test. All tests were performed by the same experimenter blinded to the treatment and/or the genotype of the animals.

In rats, the baseline mechanical and thermal sensitivity was determined prior to ddC or vehicle administration and four days after. In preliminary experiments, we showed that the maximal effect was reached four days after a single i.v. injection of 100 mg/kg ddC and persisted up to the 8th day post-injection. This time-course of behavioural effects was similar to that previously described by Joseph et al. (2004).

2.3.1. von Frey filament stimulation

Mechanical allodynia/hyperalgesia was assessed in rats using three von Frey filaments (Bioseb, Chaville, France) with bending forces of 4, 8 and 15 g. Animals were placed on an elevated grid and confined within a transparent plastic cylinder (19×29 cm). They were allowed to acclimate for 30 min before the test. The chosen filament was applied to the mid-plantar area of each hind paw until the filament just bent. A series of 10 stimuli was applied alternately to the right and left paws. Only robust and immediate withdrawal responses were considered as positive. A prolonged licking of the paw was also considered as positive. Responses from both hind paws were pooled.

In mice, von Frey filaments were also used to determine the baseline mechanical sensitivity prior to ddC or vehicle administration. The protocol described above for rats was adapted for mice using a grid with thinner mesh and smaller plastic cylinders (10×15 cm). Three ascending bending force von Frey filaments of 0.04, 0.16 and 0.4 g were successively applied. To follow the development of neuropathy, animals were tested four days after the first 500 mg/kg ddC administration. Then mice received a second 500 mg/kg ddC injection and were further tested three and five days after the second injection (seven and nine days after the first one, respectively).

2.3.2. Randall–Selitto paw-withdrawal test

Mechanical hyperalgesia in rats was assessed by measuring the paw-withdrawal threshold using the Ugo Basile analgesimeter (Apelex, Passy, France) which applies a linearly increasing mechanical force to the dorsum of the hind paw. The right and left hind paws were alternately tested at 5 min intervals. Five measures for each hind paw were performed. The mechanical threshold was defined as the mean score (5X2), expressed in arbitrary units.

2.3.3. Cold behavioural testing

Thermal allodynia was evaluated by the acetone test. Rats were placed on an elevated grid, a drop (0.1 ml) of acetone was applied at the centre of the plantar face of the hind paw and a stopwatch was started. Responses were monitored during 1 min after acetone application and were graded according to a 4-point scale, as previously described by Flatters and Bennett (2004): 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking (>2) of the paw; 3, repeated flicking of the paw with licking directed at the ventral side of the paw. Acetone was applied alternately five times to each paw and the responses scored. Cumulative scores were then obtained by summing the ten scores for each rat, the minimum score being 0 (no response to any of the ten trials) and the maximum possible score being 30 (repeated flicking and licking of paws on each of the 10 trials).

2.4. Tissue preparation

Four days after ddC or vehicle injection, rats were anaesthetized with pentobarbital (60 mg/kg i.p.) and then perfused intracardially with 100 ml of 0.9% NaCl containing 0.1% sodium nitrite followed by 800 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 (PB). Lumbar spinal cord and DRGs were dissected and post fixed overnight in the same fixative at room temperature or at 4 °C, respectively. Spinal cord sections (30 μm-thick) were made using a vibratome. Lumbar DRGs were transferred into PB containing 20% sucrose for 24 h, embedded in Tissue freezing medium® (Jung, Leica instruments, Nussloch, Germany) and then frozen on a metal plate cooled with dry ice. They were stored at −20 °C until use. Before histochemical analyses, L4 DRGs were serially cut into 14 μm-thick sections alternatively placed onto five Super-Frost® Plus slides (Menzel–Glaser, Braunschweig, Germany). Each glass slide included representative series of one out of five sections from the whole DRG.

At the end of the protocol (9 days after the first ddC injection), mice were perfused intracardially, under deep pentobarbital anaesthesia (60 mg/kg i.p.), with 100 ml of 4% PFA in 0.1 M PB, pH 7.4. Lumbar spinal cord sections were obtained as previously described for rats.

2.5. Morphological analysis of rat DRG mitochondria by electron microscopy

The morphology of ddC-treated ($n = 12$) L5 DRG was studied by electron microscopy. After post-fixation in 4% PFA, DRGs were washed in PB. They were then transversally cut in two blocks to allow a better penetration of the reagent and postfixed for 10 min at room temperature with 1% osmium tetroxide (Heraeus Chemicals South Africa, Port Elisabeth, South Africa) in PB. After a 10 min wash in PB, they were dehydrated in graded alcohol baths and successively incubated in alcohol/araldite (v/v) for 1 h at 37 °C, araldite (Fluka, Steinheim, Switzerland) for 15 h at 37 °C, and araldite supplemented with 2% accelerator (Fluka) for 3 h at 37 °C. Hemi-DRGs were then embedded in araldite (2 days at 60 °C). Silver ultrathin sections (about 70 nm-thick) were made using an ultramicrotome (LKB, Bromma, Sweden) at two different levels of the DRG after a semi-thin section to

verify the level. Ultrathin sections were collected on copper grids. They were counterstained with lead citrate for 10 min at room temperature in a drying chamber and examined using a JEM-100-CX II (JEOL, Peabody, MA, USA) electron microscope. In order to determine which type of DRG cells was affected by ddC, the number of small diameter ($<1000\ \mu m^2$), or large cell bodies ($>1000\ \mu m^2$) containing altered mitochondria was counted in ultrathin sections from 17 ddC-treated rats. The percentage of each cell type containing altered mitochondria was determined. The morphology of mitochondria was also observed in four control animals.

2.6. TUNEL assay and cell counting in DRG

To detect apoptotic cells in DRG we used a TUNEL (Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP Nick End Labelling) detection kit (Roche diagnostic, France). DRG cryostat sections (14 μm-thick) were preincubated in 0.02 M phosphate buffer (pH 7.4) containing 0.9% NaCl (PBS), 0.1% sodium citrate and 0.1% Triton X-100 for 30 min at 4 °C. After extensive washing, sections were incubated for 15 min at room temperature with TdT buffer (200 mM potassium cacodylate, 25 mM Tris–HCl pH 6.6) containing 1 mM $CoCl_2$. The enzymatic reaction was carried out in TdT buffer containing 2.5 mM $CoCl_2$, 5 μM biotinylated dUTP and TdT enzyme (1.33 U/μl) for 60 min at 37 °C. The reaction was stopped by extensive washing in PBS. Sections were then incubated with 1/1000 streptavidin-Cy3 (Sigma, Saint Quentin Fallavier, France) diluted in PBS during 45 min at 37 °C, in the dark. After washing in PBS, slices were coverslipped using Vectashield (Vector, Burlingame, USA) containing DAPI (Di Aminido Phenyl Indol) to label the nuclei. To obtain a positive control, some sections were treated for 15 min at 37 °C with 1 μg/ml DNAse I (Roche diagnostic, France) diluted in DNAse buffer (40 mM Tris–HCl, 10 mM $MgSO_4$, 1 mM $CoCl_2$, pH 8.8) prior to the enzymatic reaction.

Quantification of the total DRG cell population was performed in NaCl-treated ($n = 3$) and ddC-treated ($n = 3$) L4 DRG. DAPI-labelled sensory neuron nuclei were counted on ten randomly chosen 14 μm-thick sections. Results are expressed as mean number nuclei per $mm^2 \pm$ SEM.

2.7. Immunocytochemistry

2.7.1. 5-HT2AR antibodies

Two different rabbit anti-5-HT2AR antibodies were used. Both were directed against the 22–41 amino acid N-terminal sequence of the rat receptor (LGDGPRLYHNDFNSR-DANTS). For rat spinal cord and DRG immunolabelling, we used rabbit polyclonal antibodies purchased from Diasorin (ref 24288, Antony, France). Control Western blotting revealed a single 53 kD band corresponding to the 5-HT2AR. In addition, we previously demonstrated the specificity of these antibodies in immunocytochemistry using conventional control experiments (adsorption control with the synthetic peptide) and by the absence of labelling in 5-HT2AR−/− mice, whereas wild-type mice of the same genetic background exhibited a labelling distribution comparable to that observed in rats (Doly et al., 2004). For mouse spinal cord, we used rabbit polyclonal anti-5-HT2AR antibodies from Sigma (ref S4812, Saint Quentin Fallavier, France). The labelling was totally absent in 5-HT2AR−/−mice (see Fig. 10).

2.7.2. 5-HT2AR immunolabelling in rat spinal cord

Lumbar spinal cord sections from control and treated animals of the same series (treated with ddC or saline, tested for nociceptive behaviour and perfused together) were simultaneously processed. Immunocytochemistry was performed on free-floating vibratome sections. Sections were preincubated for 30 min at room temperature in PBS containing 3% bovine serum albumin (PBS–BSA) and 0.4% Triton X-100 (PBS–BSA-0.4TX), then incubated overnight at room temperature with 1/100 rabbit anti-5-HT2AR IgG antibodies (Diasorin) in PBS–BSA-0.4TX. After extensive washing in PBS–BSA, sections were incubated for 1 h with 1/250 biotinylated anti-rabbit IgG (Vector, Burlingame, USA) in PBS. Sections were then washed in PBS and incubated for 1 h in the avidin–biotin-peroxidase complex (Vector) diluted (1/200) in PBS. After 2×5 min washes in PBS and a 10 min wash in 0.05 M Tris–HCl, pH 7.4, sections were incubated in Tris–HCl supplemented with 0.04% (w/v) 3,3,-diaminobenzidine (Sigma) and 0.01% (v/v) hydrogen peroxide. The reaction proceeded at 20 °C under light microscope control and was stopped after a maximum of 15 min of incubation by washing in Tris–HCl buffer. Sections from one treated and one control animal were processed in parallel with the same revelation time in DAB. Sections were then washed in PBS and mounted onto glass slides in glycerol–PBS (v/v).

2.7.3. 5-HT2AR immunolabelling in mouse spinal cord

Lumbar spinal cord (L4) sections from 5-HT2AR−/−, 5-HT2AR+/+ and 5-HT2AR+/− control mice (injected with the vehicle) were simultaneously processed for immunocytochemistry. Preincubation and incubation were performed as described above for rat spinal cord sections except that we used the rabbit polyclonal anti-5-HT2AR antibodies from Sigma (1/100). As second antibody we used biotinylated anti-rabbit goat antibodies (1/250, 1 h at room temperature, Vector), followed by streptavidin-Cy3 (1/3000, 2 h at room temperature, Sigma).

2.7.4. 5-HT2AR immunolabelling in rat DRG

Immunocytochemistry was performed on cryostat sections mounted on glass slides. One slide from a ddC-treated rat and one slide from a control rat, each corresponding to one out of five sections from the whole DRG, were processed in parallel. Sections were preincubated in PBS–BSA containing 0.2% Triton X-100 (PBS–BSA–0.2TX) and then incubated for 3 days at room temperature with 1/200 rabbit anti-5-HT2AR antibodies (Diasorin) diluted in PBS–BSA–0.2TX. Sections were then incubated for 2 h in 1/2000 Alexa 555 anti-rabbit IgG (Molecular Probe, Invitrogen, Cergy-Pontoise, France). They were then washed in PBS and mounted in Vectashield mounting medium containing DAPI.

2.8. Quantification of 5-HT2A receptor immunoreactivity

2.8.1. 5-HT2AR immunolabelling in rat spinal cord

5-HT2AR immunolabelling quantification in rat spinal cord and DRG was performed by an experimenter blinded to the treatment of the animals. Spinal cord sections from

one series (one control and one ddC-treated rat) were successively photographed with the same camera parameters with a Leica light microscope (DMRB, Leica, Rueil-Malmaison, France) using a Q-imaging micropublisher camera piloted by the Q-capture system (Q-imaging, Burnaby, British Columbia, Canada). Images were then analysed using the ImageProPlus software (Media Cybernetics, Silver Spring, MD, USA). Ten sections (20 dorsal horns) of L4 spinal cord from four control and four ddC-treated rats were quantified. A shading correction was performed on spinal cord images. For each picture, five different regions were counted. Layer II was arbitrarily delimited as the most immunolabelled layer with our antibody. Layer I was determined as the upper adjacent layer. Layer III, located ventrally to layer II, was counted only in its lateral part. The lateral part of layer V, easily identified by its fascicular organisation, was counted. Finally, a region localized in the medial border of the grey matter in the neck of the dorsal horn, corresponding to a ventral extension of lamina IV (IVv) (Molander et al., 1984), was also counted (see Fig. 5a). Grey levels were measured on 12-bit images (0–4095). For each section, the mean grey level was also measured in a region localized in the white matter between dorsal horns characterized by an absence of immunoreactivity (see Fig. 5a). This measure was used for background correction. For each layer, the mean grey level from a control animal (Dc) was subtracted from the mean grey level of a ddC-treated animal (Dt) from the same series. The differences of grey level (Dt–Dc) were used for statistical analysis and then expressed as percentage of enhanced grey level (see Fig. 5b).

2.8.2. 5-HT2AR immunolabelling in rat DRG

In order to avoid fluorescence photo-bleaching, DRG-immunolabelled sections were not observed before the quantification. DRG sections from one series, comprising a control and a ddC-treated rat, were successively photographed with fluorescence light microscope (Axiovert, Zeiss, Le Pecq, France) using the AxoVision (Zeiss) software. Mosaic images of each section were created. The exposure time was determined for the ddC-treated DRG and then applied to the control DRG. 5-HT2AR-positive neurons were counted with the ImageProPlus software (Media Cybernetics, Silver Spring, MD, USA) by an experimenter blinded to the treatment of the animals and the mean diameter of labelled neurons was measured. Only neurons having their nucleus (labelled with DAPI) in the plane of the section were included. One slide of a control animal and one slide of a treated animal, each containing every fifth section of an entire DRG, were analysed in parallel. Four controls and four ddC-treated animals were used for quantification.

2.9. Statistical analysis

One-tail unpaired *t*-tests were used to compare control and ddC-treated behavioural results, before and four days after drug or vehicle (NaCl) administration and one-tail paired *t*-tests were used to compare ddC-treated behavioural results before and four days after ddC injection.

For quantification of 5-HT2AR in spinal cord, ddC-induced changes in 5-HT2AR immunoreactivity (expressed as difference of mean grey levels Dt-Dc) were analysed with one-tail *t*-test. For quantification of mean number and mean diameter of 5-HT2AR positive neurons in DRG, the one-tail unpaired *t*-test was used to compare control and ddC-treated animals. The same test was used for comparing the number of DAPI nuclei. Significance was accepted at $p < 0.05$.

In the study of the effect of MDL 11,939 on mechanical withdrawal, one-tail paired *t*-tests were used to compare withdrawal frequency to von Frey stimulation in all experimental groups (ddC/aCSF or ddC/1–100 μM MDL 11,939) before and four days after ddC injection. For each ddC/MDL 11,939 treated groups, von Frey withdrawal frequencies at 30, 60 and 120 min after MDL 11,939 injection were compared to pre-MDL 11,939 values (Time 0) with a repeated measures one-way ANOVA and Bonferroni post hoc test. One-way ANOVA with Bonferroni post hoc test was used to compare AUCs (areas under the curve) corresponding to MDL 11,939 (1–100 μM) and aCSF treatments.

In the study of mechanical withdrawal in 5-HT2AR+/+ mice, repeated measures one-way ANOVA and Bonferroni post hoc test were used to compare withdrawal frequency to von Frey stimulation before and after ddC-injection. At each time post-injection, the response frequencies were compared between the experimental groups (5-HT2AR+/+, 5-HT2AR−/− and heterozygous mice) using one-way ANOVA with Bonferroni post hoc test.

3. Results

3.1. Morphology of rat DRG mitochondria and TUNEL assay

The electron microscopic examination of the L5 DRG in ddC-treated rats, four days after treatment, demonstrated mitochondrial injury in numerous neuronal cell bodies (Fig. 1). Altered mitochondria were characterized by a drastic swelling and a disappearance of the cristae (Fig. 1C and F). These modifications involved almost all mitochondria in affected cell bodies, which were more frequently small and medium sized nociceptive cell bodies ($<1000\ \mu m^2$) than larger ones ($>1000\ \mu m^2$). However, some large cell bodies, presumably those corresponding to $A\beta$ fibres, were also affected (Fig. 1F), but the severity of the mitochondrial injury was generally less important. The number of cell bodies exhibiting injured mitochondria varied among animals, but all treated animals were affected. Quantification showed that $57.7 \pm 4.3\%$ ($n = 1350$ cells in 17 DRG) of small neurons but only $24.8 \pm 4.5\%$ ($n = 1070$ cells in n=17 DRG) of large neurons contained injured mitochondria. Vehicle-treated animals were never affected (not shown). In the lumbar dorsal horn, no mitochondrial alteration was observed in central terminals of DRG cells. The central terminals of type II glomeruli, corresponding to $A\delta$ fibre terminals and characterized by their enrichment in mitochondria (da Silva and Coimbra, 1985), were not modified. In addition, no morphological modification was observed in dorsal horn cell bodies (not shown).

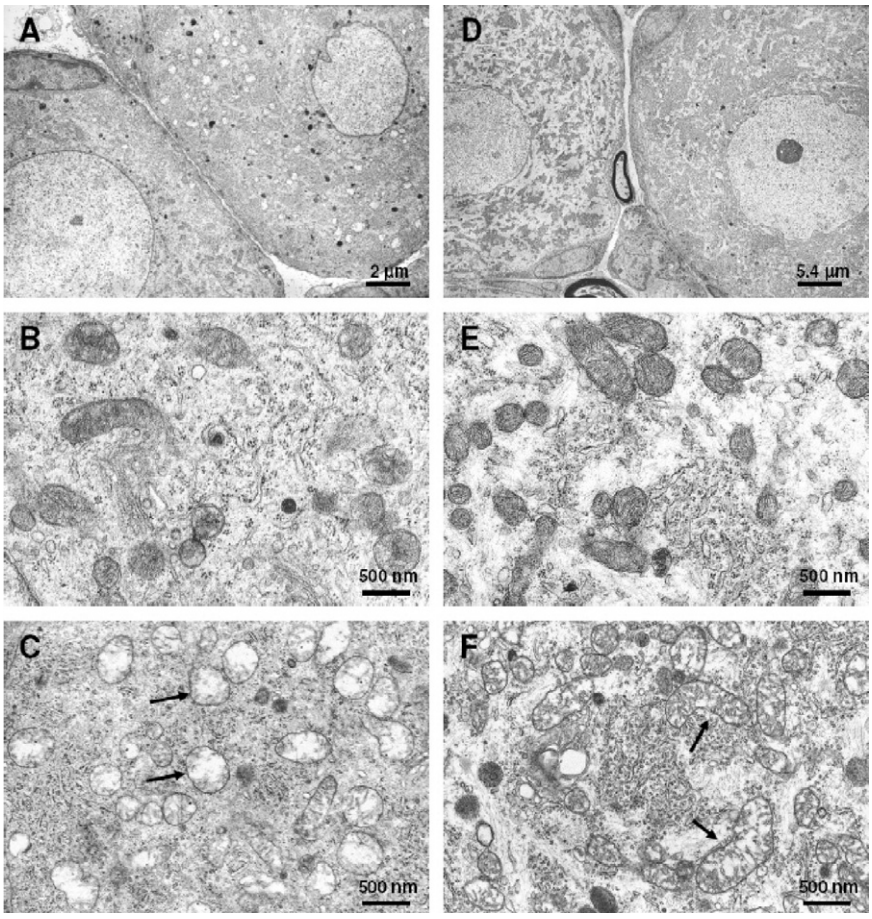

Fig. 1. Ultrastructure of rat lumbar DRG neurons four days after a single i.v. injection of 100 mg/kg ddC. (A) Shows two small DRG neurons, the left one contains normal mitochondria (enlargement of the cytoplasm in (B)), whereas in the right one (large magnification in (C)), all mitochondria are swollen and most cristae are injured (arrows). (D) Shows two large DRG neurons, the left one (enlargement in (E)) contains normal mitochondria whereas the right one (enlargement in (F)) contains injured mitochondria (arrows). Notice that the mitochondria appear less injured than in the small DRG neuron (in (C)).

TUNEL assay performed on lumbar DRG sections showed a complete absence of TUNEL positive nuclei four days after a single injection of 100 mg/kg ddC (Fig. 2A), whereas DNAse I-treated sections (positive control) exhibited strong TUNEL labelling (Fig. 2B). In addition, no TUNEL labelling was observed in the dorsal horn (not shown). Quantification of the whole DRG neuronal population showed that the density of cell bodies was not modified four days after a single ddC injection (291.0 ± 1.7 cell bodies/mm^2) compared to the vehicle-treated group (289.7 ± 12.5 cell bodies/µm^2) receiving NaCl injection ($n = 3$ in each group). A Student's *t*-test indicated that the mean number of DAPI-labelled nuclei was not statistically different.

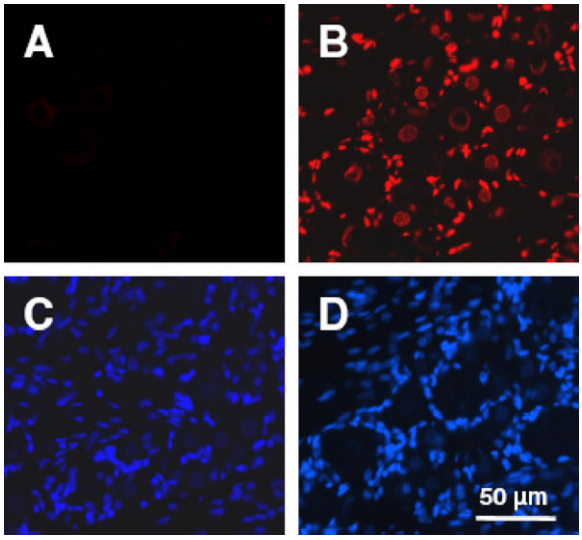

Fig. 2. TUNEL assay in lumbar DRG sections four days after a single i.v. injection of 100 mg/kg ddC. (A) TUNEL labelling in DRG from ddC-treated rat is totally absent, while a TUNEL positive DRG control treated with DNAse I shows a strong staining (B). DAPI counterstaining is shown in (C,D).

3.2. Behavioural tests in rats

3.2.1. von Frey filament stimulation

Before vehicle or ddC injection, the baseline paw-withdrawal frequency after mechanical stimulation

with von Frey filament of 4 g bending force was not significantly different in both groups of animals ($2.7 \pm 1.4\%$ for the vehicle-treated group and $1.7 \pm 1.1\%$ for the ddC-treated group) (Fig. 3a). Likewise, at day 0, the baseline paw-withdrawal frequency after stimulation with 8 and 15 g von Frey filaments was not significantly different in vehicle and ddC-treated animals ($3.6 \pm 1.5\%$ and $6.7 \pm 2.8\%$, respectively, for 8 g, $13.6 \pm 4.1\%$ and $16.7 \pm 3.8\%$, respectively, for 15 g) (Fig. 3b and c). Four days after a single injection of 100 mg/kg ddC, responses to 4, 8 and 15 g von Frey filament stimulation were significantly enhanced ($20.8 \pm 4.2\%$, $30.8 \pm 5.6\%$ and $46.7 \pm 7.6\%$, respectively) compared to baseline responses obtained before injection and to responses of vehicle-treated rats (0%, $4.6 \pm 1.6\%$ and $12.7 \pm 2.4\%$, respectively) (Fig. 3a–c). DdC-induced enhanced responses to the 4 g von Frey filament are best described as allodynia (pain from a normally innocuous stimulus), because baseline paw-withdrawal frequency was <5%. Responses to the 15 g von Frey filament are best described as hyperalgesia (heightened pain response from a normally painful stimulus) because control animals withdraw from this stimulus 5–15% of the time. Responses to 8 g are intermediate.

3.2.2. Randall and Selitto paw-withdrawal test

At day 0, before injection, the baseline threshold for mechanical nociceptive stimulation using the Ugo-Basile analgesimeter was not significantly different between vehicle and ddC-treated groups (5.8 ± 0.1 and 5.9 ± 0.1 A.U., respectively) (Fig. 3d). Four days after injection, the paw-withdrawal threshold was significantly decreased in the ddC-treated group (4.9 ± 0.2 A.U.) compared to their baseline value (5.9 A.U.) and to vehicle-treated rats (6.1 ± 0.2 A.U.), indicating that a single intravenous injection of 100 mg/kg ddC induced a mechanical hyperalgesia.

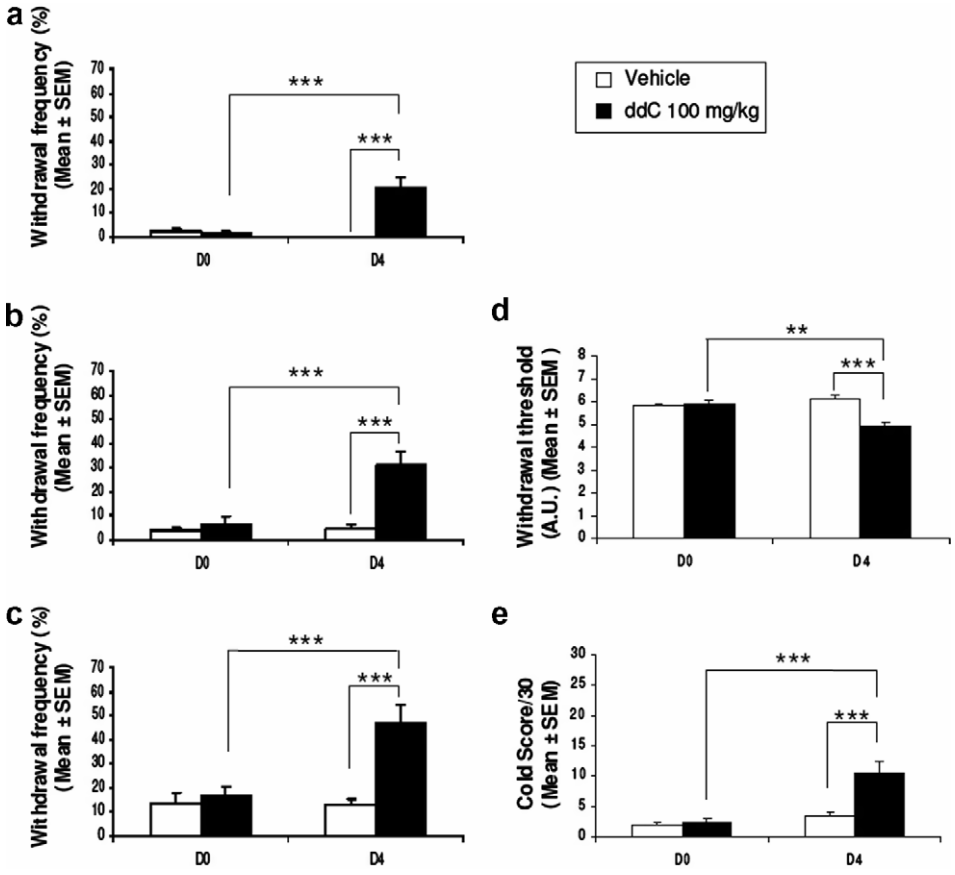

Fig. 3. Effect of a single i.v. injection of 100 mg/kg ddC on pain behaviour. (a–c) Paw withdrawal reflex to von Frey filament stimulation. Response frequencies are shown before the treatment at day 0 and four days (day 4) after a single injection of ddC (black bars) or vehicle (empty bars). Data are expressed as means ± SEM response frequency. The mean response frequency at day 4 is significantly increased following stimulation with the von Frey filaments of 4 g (a), 8 g (b) and 15 g (c) bending force compared to vehicle-treated animals and to stimulation frequency before treatment. (d) Paw-withdrawal threshold using an Ugo-Basile analgesimeter (Randall and Selitto test). Data are expressed as means ± SEM withdrawal threshold in arbitrary units (A.U.). The paw-withdrawal threshold is lower at day 4 in ddC-treated rats compared to baseline threshold and to vehicle-treated rats. (e) Effect of ddC treatment on cold stimulation (acetone test). Data are expressed as mean cold score ± SEM (see details in Section 2). $n = 10$/group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

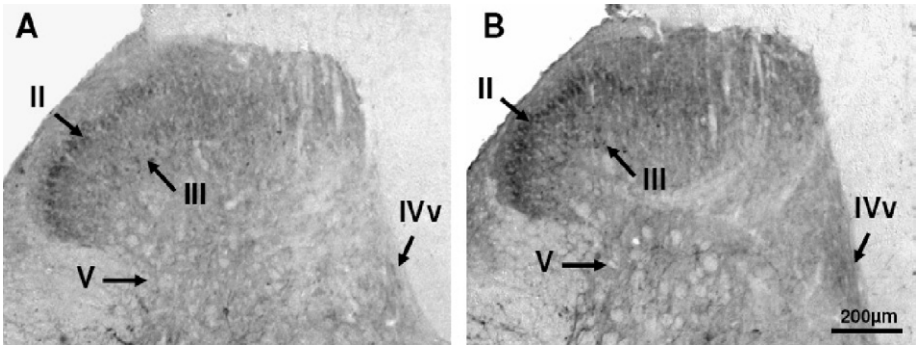

Fig. 4. 5-HT2AR immunoperoxidase labelling in the dorsal horn four days after a single i.v. injection of 100 mg/kg ddC. The ddC-treated rat (B) shows a higher density of labelling throughout the dorsal horn compared to the vehicle-treated rat (A). The increased density is most evident in the superficial layers II and III but is also present in layer I and in deeper layers V and IVv.

3.2.3. Cold behavioural testing

Before vehicle or ddC injection, cold scores were $2.0/30 \pm 0.6$ and $2.6/30 \pm 0.6$, respectively, indicating very low responses to acetone application (Fig. 3e). Four days after injection, the mean cold score was significantly increased in the ddC-treated group ($10.7/30 \pm 1.9$) compared to the mean cold score of the vehicle-treated group ($3.5/30 \pm 0.6$). Therefore, this increase of the cold score (about threefold) indicated that ddC treatment induces a cold allodynia.

3.3. 5-HT2AR expression in the rat dorsal horn of the lumbar spinal cord

The distribution of 5-HT2AR-immunoreactivity in the dorsal horn of the rat spinal cord was heterogeneous, with a higher density of labelling in the superficial layers, in particular in the lateral part of layer II (Fig. 4A). Layers III and V were also intensely labelled, whereas layers IVv was less immunoreactive. The general distribution of 5-HT2AR labelling corresponds to our previous observations (Doly et al., 2004). Animals analyzed four days after a single intravenous administration of 100 mg/kg ddC showed denser 5-HT2AR-immunoreactivity in the dorsal horn (Fig. 4B). The increased labelling density was particularly clear in layers II and III but it was also visible in other layers, particularly in layers V and IVv. Quantification of 5-HT2AR-immunoreactivity in the different layers of the dorsal horn (Fig. 5b) demonstrated a significant increase ($p < 0.01$) in layers II ($49.5\% \pm 14.6\%$), III ($57.8\% \pm 19.3\%$) and IVv ($48.3\% \pm 17.3\%$) in the ddC-treated group compared to the vehicle-treated group. The 5-HT2AR immunolabelling in layer I of the ddC-treated group exhibited a significant increase ($p < 0.05$) ($58.5\% \pm 15.5$) compared to the vehicle-treated rats. A small increase of 5-HT2AR immunolabelling was found in layer V ($19.2\% \pm 8.8\%$, $p < 0.05$). Due to variability between individuals, the significance in layer I and V was relatively low.

3.4. 5-HT2AR expression in lumbar rat DRG

In both vehicle and ddC-treated rats, 5-HT2AR-immunoreactivity was found in small and medium-sized cell bodies (Fig. 6). Labelling was mainly observed in the cytoplasm, but it was also occasionally found at the plasma membrane (Fig. 6B). The density of labelled cell bodies appeared to be enhanced in ddC-treated rats. Quantification of immunoreactive cell bodies showed that four days after a single administration of ddC

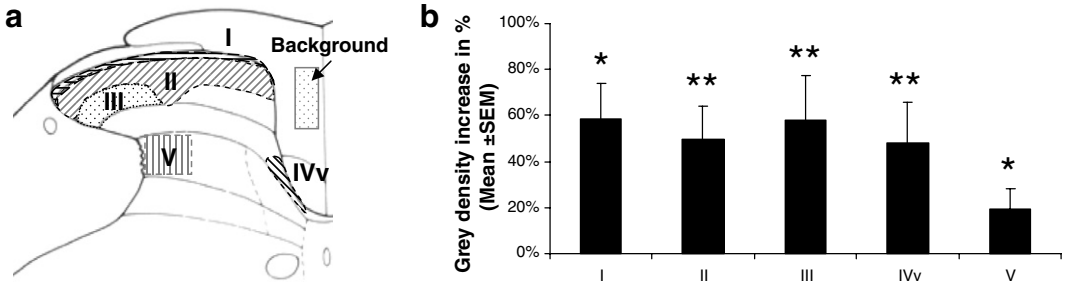

Fig. 5. Quantification of 5-HT2AR immunolabelling in the dorsal horn four days after a single i.v. injection of 100 mg/kg ddC. (a) Diagram of the subdivisions of the dorsal horn used for immunostaining quantification (see details in Section 2). (b) Graph showing the mean ± SEM of percentage of grey level enhancement of 5-HT2AR-immunoreactivity. The grey level is enhanced in all dorsal horn layers in ddC-treated rats. $n = 4$ animals/group, $n = 10$ sections/animal $^{*}p < 0.05$, $^{**}p < 0.01$.

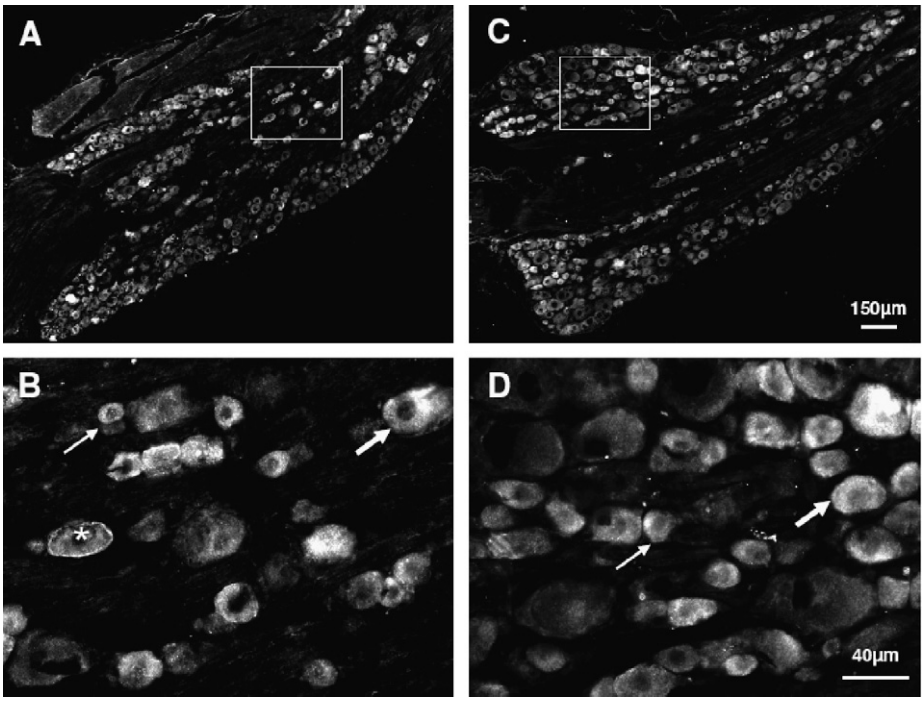

Fig. 6. 5-HT2AR immunofluorescence labelling in L4 DRG four days after a single i.v. injection of 100 mg/kg ddC. 5-HT2AR immunoreactivity in DRG from vehicle (A,B) and ddC-treated rats (C,D) is comparable, with a large predominance in small (thin arrows) and medium cell bodies (large arrows). The density of labelled soma appears to be higher in ddC-treated rats. (B,D) Correspond to enlargements of the frames in (A,C), respectively.

(100 mg/kg, i.v.), the number of positively-stained 5-HT2AR neurons in DRG was significantly increased by 30.7% (Fig. 7). The number of immunoreactive cell bodies in every fifth section of the entire L4 DRG (corresponding to 15–20 14 µm-thick sections per animal) was 433 ± 32 in vehicle-treated animals ($n = 4$) and 566 ± 53 in ddC-treated animals ($n = 4$, $p < 0.05$). The size distribution of 5-HT2AR positive neurons was not modified by ddC-treatment (Fig. 7). The mean diameter of 5-HT2AR-positive DRG neurons of vehicle and ddC-treated animals was 34.9 ± 1.9 and 34.0 ± 0.7 µm, respectively. In ddC-treated animals, the number of labelled cell bodies was significantly increased in the class size comprised between 29 and 30 µm diameter ($n = 4$ animals/group, $n = 15$–20 sections/animal, $^{**}p < 0.01$).

3.5. Effect of a 5-HT2AR antagonist on mechanical withdrawal in ddC-treated rats

Epidural injection of the 5-HT2AR antagonist MDL 11,939 significantly attenuated mechanical allodynia and hyperalgesia induced by ddC-treatment (Fig. 8a–c). The maximal effect was obtained 30 min after an epidural injection of 25 µl of 100 µM MDL 11,939 which significantly reduced ($p < 0.01$) paw withdrawal frequency to

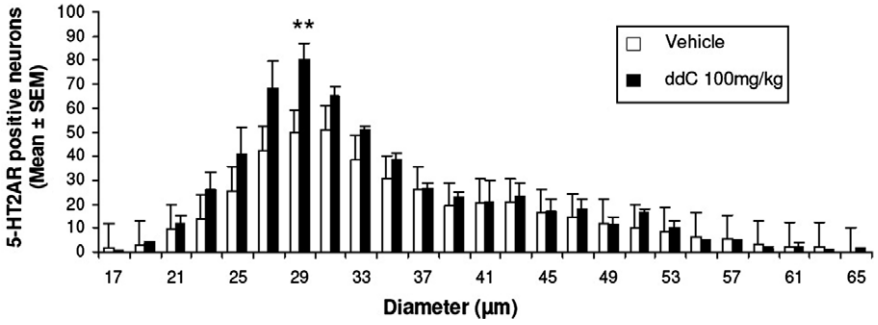

Fig. 7. Size distribution histogram of 5-HT2AR-immunoreactive cell bodies in L4 DRG four days after a single i.v. injection of 100 mg/kg ddC. Data are expressed as mean number (±SEM) of 5-HT2AR-positive neurons classified by diameter µm (2 µm steps). The size distribution of labelled cell bodies is similar in vehicle and ddC-treated animals. The number of immunoreactive cell bodies is significantly increased in the class size corresponding to 29–30 µm diameter ($n = 4$ animals/groups, $^{**}p < 0.01$).

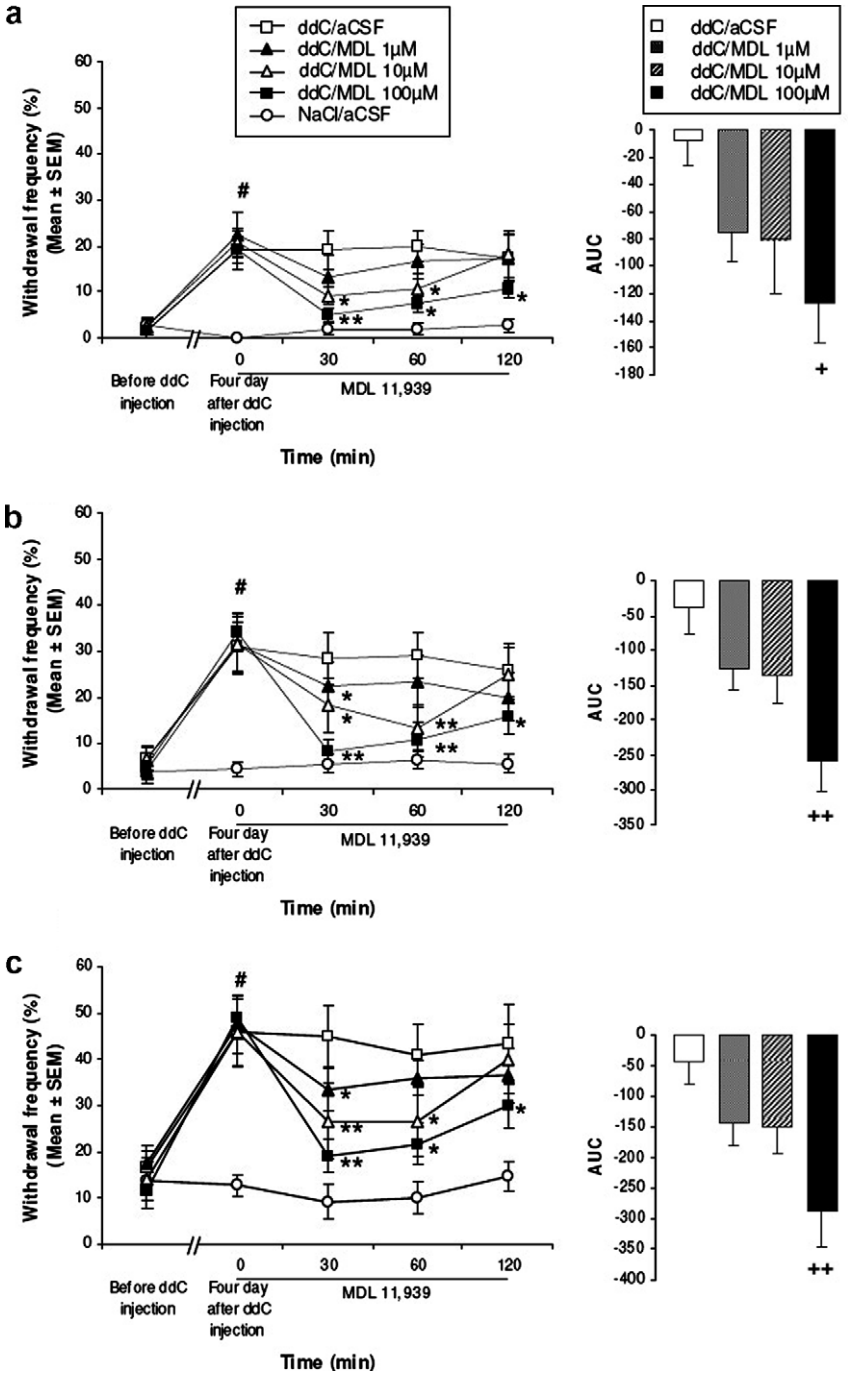

Fig. 8. Effect of a 5-HT2AR antagonist on mechanical withdrawal in ddC-treated rats. Fours days after a single 100 mg/kg ddC or vehicle i.v. injection, rats received an epidural injection of MDL 11,939 (1–100 μM), or its vehicle (aCSF), then paw withdrawal reflex to 4 g (a), 8 g (b) or 15 g (c) von Frey filament stimulation was measured 30, 60 and 120 min later. Each point is the mean ± SEM of 12 ddC/aCSF, 12 ddC/1 μM MDL, 12 ddC/10 μM MDL, 12 ddC/100 μM MDL, 11 vehicle/aCSF independent determinations (10 measurements by rat). The first point corresponds to measures made before ddC or vehicle injection, the second (time 0) to measures made just prior to MDL or aCSF injection. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to corresponding values before MDL injection (repeated measures one-way ANOVA with Bonferroni post hoc test). $^{\#}p < 0.001$, compared to corresponding values before ddC injection (*t*-test). The right hand panel indicates the area under the curve (AUC) calculated from curves shown on the left panel. $^{+}p < 0.05$, $^{++}p < 0.01$ curve after 1–100 μM MDL injection compared to curve after aCSF injection (One-way ANOVA).

von Frey filament of 4, 8 and 15 g stimulation of ddC-treated rats ($19.17 \pm 2.88\%$ to $5.00 \pm 1.51\%$, $34.17 \pm 4.17\%$ to $8.33 \pm 2.41\%$, and $49.17 \pm 4.84\%$ to $19.17 \pm 3.58\%$, respectively). An injection of 25 μl of 10 μM MDL 11,939 significantly reduced paw withdrawal frequency of ddC-treated rats to von Frey filament stimulation 30 min after injection: $20.83 \pm 3.13\%$ to $9.17 \pm 1.93\%$ ($p < 0.05$) for the filament of 4 g bend-

ing force, 31.67 ± 6.01% to 18.33 ± 5.88% ($p < 0.05$) for the 8 g filament and 45.83 ± 7.23% to 26.67 ± 8.29% ($p < 0.01$) for the 15 g filament. The lower dose (1 μM MDL) significantly ($p < 0.05$) reduced the paw withdrawal frequency to stimulation with the von Frey filament of 15 g bending force, 30 min after injection (47.50 ± 6.17% to 33.33 ± 4.66%). The comparison of the areas under the curves (AUC) calculated from 0 to 120 min after MDL or aCSF injection for each group of animal, showed that the AUC obtained for ddC-treated rats injected with 100 μM MDL 11,939 was significantly higher compared to ddC-treated animals injected with aCSF ($p < 0.05$ for filament of 4 g bending force, $p < 0.01$ for 8 g and 15 g filaments) (Fig. 8, right panel). It indicated that the reduction of paw withdrawal frequency after MDL epidural injection was dose-dependent.

On its own, administration of MDL 11,939 did not modify mechanical responses to von Frey filament stimulation in naïve rats ($n = 8$ rats, 10 measurements by rat). Thirty minutes (T30) and 60 min (T60) after epidural injection of 25 μl of 100 μM MDL injection, the response frequencies to von Frey filaments stimulation were not statistically modified compared to values measured before MDL injection (6.3 ± 3.8% at T0, 5.0 ± 1.9% at T30, 5.0 ± 1.9% at T60 for filament 8 g; 15 ± 5% at T0, 22.5 ± 8% at T30 and 16.3 ± 2.6% at T60 for filament 15 g; 26.3 ± 4.2% at T0, 27.5 ± 6.2% at T30 and 21.3 ± 5.2% at T60 for filament 26 g).

3.6. Neuropathy induced by ddC depends on 5-HT2AR expression in mouse

Before ddC injection, the baseline paw-withdrawal frequency after mechanical stimulation with the different von Frey filaments was not significantly different in 5-HT2AR+/+, 5-HT2AR−/− and 5-HT2AR+/− mice (Fig. 9). Before ddC injection, 5-HT2AR+/+ mice only poorly responded to von Frey filament of 0.04 g bending force (response frequency 2.9 ± 1.8%) (Fig. 9a), whereas their withdrawal frequency reached 14.3 ± 5.3% and 35.7 ± 7.2% to 0.16 g and 0.4 g von Frey filaments, respectively (Fig. 9b and c). Four days after ddC injection, the response frequencies of 5-HT2AR+/+ mice to 0.16 and 0.4 g von Frey filaments were significantly enhanced (34.3 ± 6.9% and 60.0 ± 8.2%, respectively), whereas the response frequency to the smaller filament was unchanged. At day 4, mice received a second ddC injection. Three and five days later (days 7 and 9), response frequencies to 0.16 and 0.4 g von Frey filaments were further significantly ($p < 0.01$) enhanced (55.7 ± 3.7% at day 9 compared to 14.3 ± 5.3% at day 0 for filament 0.16 g and 82.9 ± 5.2% compared to 35.7 ± 7.2 at day 0 for the filament 0.4 g). Response frequency to the von Frey filament of 0.04 g bending force was significantly higher ($p < 0.05$) than that observed at day 0 (31.4 ± 4.0% at day 9 compared to 2.9 ± 1.8% at day 0). These data indicated that the ddC-treatment induced both a mechanical hyperalgesia and allodynia in wild-type mice.

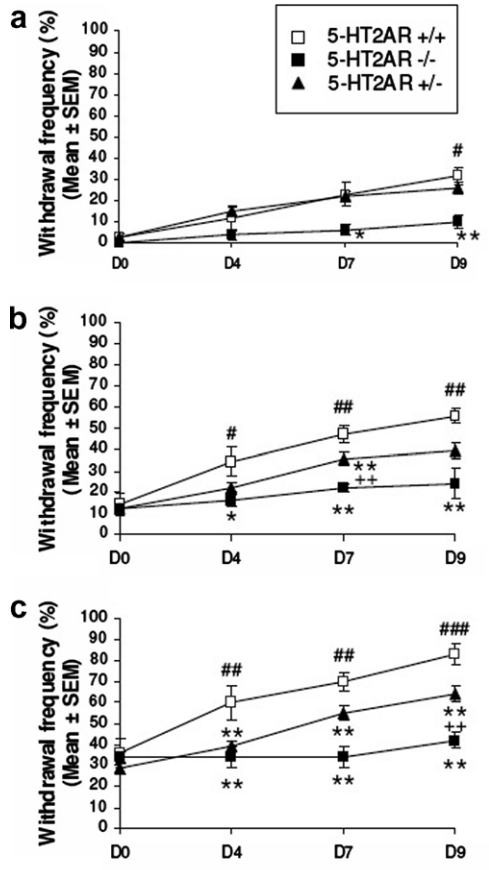

Fig. 9. Neuropathy induced by ddC depends on the genetic expression of the 5-HT2AR in mice. Response frequencies to von Frey filaments of 0.04 g (a), 0.16 g (b) and 0.4 g (c) stimulations were measured at day 0 before ddC injection (500 mg/kg i.p), at day 4, before the second ddC injection, at day 7 and 9, 3 and 5 days after the second injection. Each point is the mean ± SEM of 10 measurements by mice on 5-HT2AR−/− ($n = 5$), 5-HT2AR+/+ ($n = 7$), 5-HT2AR+/− ($n = 14$) mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.05$, comparison of withdrawal frequencies of 5-HT2AR+/+ mice to von Frey filaments compared to withdrawal frequencies at day 0 (repeated measures one-way ANOVA). $p < 0.05$, $^{**}p < 0.01$, values from 5-HT2AR−/− or 5-HT2AR+/− mice compared to corresponding values obtained in 5-HT2AR+/+ mice (one-way ANOVA). $^{++}$ $p < 0.01$, values from 5-HT2AR+/− mice compared to 5-HT2AR−/− mice (one-way ANOVA).

By contrast, after the two injections of ddC, paw withdrawal frequency of the 5-HT2AR−/− mice to mechanical stimulation with the different von Frey filaments remained statistically unchanged. Moreover, response frequencies of 5-HT2AR−/− mice at day 9 post-injection to all three von Frey filaments stimulations were statistically different ($p < 0.01$) from those of 5-HT2AR+/+ mice (31.4 ± 4.0% and 10.0 ± 3.2%,

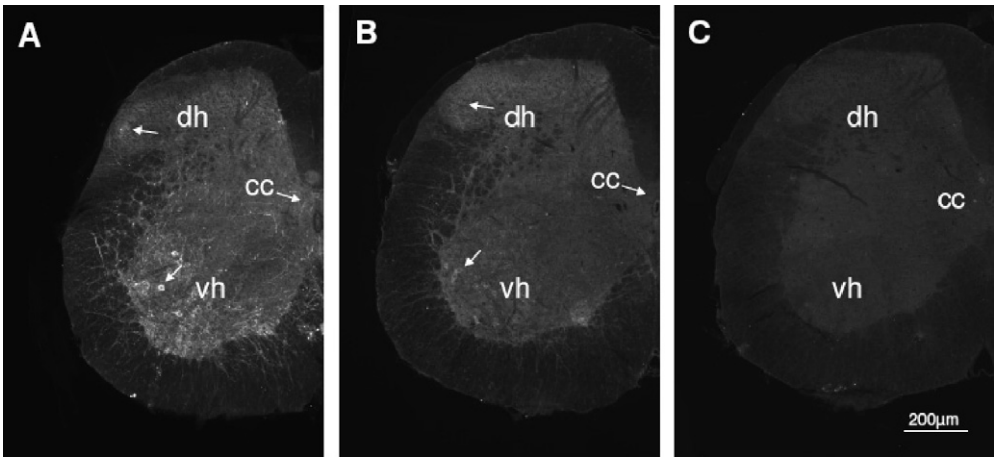

Fig. 10. 5-HT2AR immunolabelling in mouse spinal cord (A) 5-HT2AR immunolabelling in a lumbar spinal cord from a 5-HT2AR+/+ mouse. The densest staining is observed in the ventral horn (vh), particularly in layers IX. A dense labelling is also seen in the upper dorsal horn (dh) and in layer X around the central canal (cc). (B) A spinal cord from a 5-HT2AR+/− mouse exhibited a less intense immunoreactivity in both the dorsal and ventral horns. A moderate staining is still observed in the lateral part of the dorsal horn (arrow). Also notice the faint immunostaining of motoneurons (arrow) compared to that observed in wild-type mice in (A). (C) In a lumbar spinal cord from a 5-HT2AR−/− mouse, 5-HT2AR immunolabelling is totally absent, except for a very low background.

respectively, for 0.04 g filament; $55.7 \pm 3.7\%$ and $24.0 \pm 7.5\%$, respectively, for 0.16 g filament; $82.9 \pm 5.2\%$ and $42.0 \pm 3.7\%$, respectively, for 0.4 g filament). These results indicated that the 5-HT2AR−/− mice did not develop a painful neuropathy after ddC-treatment.

The response frequency of heterozygous mice to stimulation with the von Frey filament of 0.4 g bending force was significantly different ($p < 0.01$) at day 9 from response frequencies of 5-HT2AR+/+ and 5-HT2AR−/− mice ($64.3 \pm 3.9\%$, $82.9 \pm 5.2\%$ and $42.0 \pm 3.7\%$, respectively). The same tendency was observed after stimulation with 0.16 and 0.4 g von Frey filaments, whereas differences were not statistically different, except at day 7 for filament 0.16 g ($p < 0.01$). These results showed that 5-HT2AR+/− mice developed a less pronounced neuropathy than wild-type mice.

3.6.1. 5-HT2AR immunolabelling in the mouse spinal cord

In order to know whether this intermediate "neuropathic phenotype" might be related to the level of 5-HT2AR expression, we have compared the 5-HT2AR immunolabelling in the spinal cord from 5-HT2AR+/+, 5-HT2AR+/− and 5-HT2AR−/− mice. In 5-HT2AR+/+ mice, 5-HT2AR immunolabelling was widely distributed throughout the spinal cord (Fig. 10A). It was particularly dense in layer IX and in the ventral part of layer X around the central canal. The dorsal horn exhibited a high staining in particular in a band corresponding to layer II. The deeper layers of the dorsal horn, including the neck of the dorsal horn, displayed a moderate labelling. This pattern of immunoreactivity was comparable to that observed in the rat spinal cord (present data and Doly et al., 2004). Heterozygous mice exhibited a lower immunoreactivity in both the dorsal and ventral horns than that observed in 5-HT2AR+/+ mice (Fig. 10B), whereas 5-HT2AR immunolabelling was totally absent in 5-HT2AR−/− mice, except for a very low background uniformly distributed (Fig. 10C). These data suggested that heterozygous mice displayed an intermediate level of the receptor protein.

4. Discussion

Our results show for the first time that ddC induces direct mitochondrial toxicity in the rat. DdC-induced mitochondrial toxicity has previously been described in cultured neuronal cells (Cui et al., 1997) and in human nerves (Dalakas et al., 2001) but was not yet demonstrated in the rat despite pharmacological data showing that the mitochondrial electron transport chain is affected (Joseph and Levine, 2006). We show that after a single injection of 100 mg/kg ddC, the morphology of mitochondria is severely affected in lumbar DRG neurons. In addition, small and medium-sized neurons, presumably nociceptive, are more frequently and more severely affected than large cell bodies. Thus, the specific injury to DRG neurons, in particular to nociceptive cells, shows that a single injection of ddC is a good model of peripheral painful neuropathy in the rat. Indeed, four days after a single i.v. administration of ddC, we observed both the injury of peripheral cell bodies and the clinical signs of neuropathy, i.e. mechanical hyperalgesia and allodynia as previously observed in the comparable model in the rat (Joseph et al., 2004). Furthermore, whereas these authors only described a thermal hyperalgesia,

we also observed the appearance of a thermal allodynia in ddC-treated rats.

Despite the drastic morphological changes observed in mitochondria, we could not detect neuronal apoptosis as revealed by the absence of TUNEL labelling in the DRG. Moreover the total number of neurons was not modified by ddC treatment. These data indicate that apoptosis is not required to produce the clinical signs of neuropathy in our model. Nevertheless, it has been shown that caspase signalling pathways are involved in neuropathic pain induced by ddC (Joseph and Levine, 2004). It is possible that a longer ddC treatment may induce apoptosis in animals. However the long-term neuropathic pain observed in diabetes is not associated with apoptosis, although the level of activated caspases is high (Cheng and Zochodne, 2003). Moreover, in humans, the clinical symptoms are generally reversed by the interruption of the treatment (Blum et al., 1996), suggesting that the cellular alterations may be reversible. We also demonstrated the absence of mitochondrial injury in the dorsal spinal cord in primary afferent terminals and in spinal neurons. This is in agreement with the poor permeability of the blood brain barrier to ddC (see Roche Pharmaceutical information).

The significant up-regulation of 5-HT2AR immunoreactivity in the lumbar dorsal horn and peripheral nociceptive cells after ddC-treatment as well as the MDL 11,939-induced dose-dependent decrease of mechanical hypersensitivity induced by ddC demonstrates that the 5-HT2AR plays a crucial role in ddC-induced neuropathy at the spinal and peripheral levels.

Four days after a single injection of ddC, the number of lumbar DRG neurons exhibiting 5-HT2AR immunoreactivity is significantly increased. This can be the result of an up-regulation of the receptor, leading to the detection of a greater number of labelled cells. Alternatively, 5-HT2AR could be synthesized by a new cell population. However, this is unlikely since 5-HT2AR is expressed by a similar population of neurons in vehicle- and ddC-treated animals: small to medium-sized neurons, presumably nociceptive. Peripheral 5-HT2AR are widely involved in inflammatory-induced hyperalgesia (Abbott et al., 1996; Doi-Saika et al., 1997; Tokunaga et al., 1998; Okamoto et al., 2002; Liu et al., 2005; Wei et al., 2005; Sasaki et al., 2006). These inflammatory pain states are generally accompanied by a strong up-regulation of 5-HT2AR expression in DRG (Wu et al., 2001; Liu et al., 2005). In particular, Okamoto et al. (2002) have shown an up-regulation of 5-HT2AR mRNA (by 20%) by inflammation with complete Freund's adjuvant. By contrast they noted that axotomy or chronic constriction of a peripheral nerve did not modify 5-HT2AR mRNA levels. We observed that ddC-induced neuropathy led to a comparable up-regulation of the 5-HT2AR expression (30.7%) in peripheral nociceptive cells. Interestingly, despite strong mitochondrial impairment in nociceptive cells, there was no loss of DRG neurons after ddC treatment, in contrast with models of nerve axotomy which are most often associated with apoptosis. The important modulation of 5-HT2AR immunoreactivity observed in our model suggests that 5-HT2AR is involved in the sensitization of peripheral nociceptive neurons.

Four days after ddC injection in the rat, the increased 5-HT2AR immunolabelling density in the lumbar dorsal horn was particularly important in layers II and III but it was also significant in deeper layers. Activation of spinal 5-HT2AR has been reported to facilitate or to inhibit the transmission of nociceptive messages (Li and Zhuo, 1998; Bardin et al., 2000a,b). Several cellular mechanisms may be involved in 5-HT2AR-mediated sensitization of dorsal horn neurons. One of them involves the demasking of silent glutamatergic synapses via AMPA/5-HT2AR interactions through PDZ proteins (Li et al., 1999). The laminar distribution of 5-HT2AR in the dorsal horn (present data and Doly et al., 2004) is compatible with this hypothesis, as it exhibits close similarities with the distribution of AMPA subunit receptors (Nagy et al., 2004). Recruitment of silent AMPA synapses has been evidenced for both nociceptive-specific and wide dynamic range (WDR) neurons (Li et al., 1999) suggesting that neurons in both layers I and V–VI could be concerned by this mechanism. 5-HT2AR may also play a role in spinal sensitization by promoting "plateau potentials" (Morisset and Nagy, 1998; Perrier et al., 2002), which could be related with the "wind-up" phenomenon described when WDR neuron activity is repeatedly evoked by noxious electrical stimulation. We have also shown that 5-HT2AR immunoreactivity is enhanced in layer II after ddC treatment. Layers II contains nociceptive primary afferent terminals and several classes of excitatory and inhibitory interneurons (Willis and Coggeshall, 1992) that modulate layer I projection neurons (Light, 1988). The enhanced 5-HT2AR immunoreactivity in layer II may be due to an up-regulation of the receptor either in primary afferent terminals or in interneurons. Taken together, these findings show that the 5-HT2AR is involved in a wide central sensitization of dorsal horn neurons and in a peripheral sensitization of nociceptive neurons. The mechanism leading to the 5-HT2AR expression is unknown but several data suggest that an auto-induction may occur (Du et al., 1994).

The dose-dependent decrease of ddC-induced mechanical hyperalgesia/allodynia following epidural administration of MDL 11,939 further demonstrates that the 5-HT2AR plays a crucial role in ddC-induced neuropathy at the spinal and/or peripheral levels. The epidural administration of MDL 11,939 may lead to a diffusion of the drug to both spinal cord and DRG cells. The observed effect may thus be the result of MDL 11,939 binding to various spinal neurons and

to nociceptive DRG cells that express the 5-HT2AR in their plasma membrane (unpublished results). Moreover, the 5-HT2AR is expressed by neurons of the whole spinal cord, including motoneurons and preganglionic neurons, where it modulates motor and autonomic functions (see Schmidt and Jordan, 2000; McLean et al., 2007). However, we verified that MDL 11,939 does not modify mechanical responses to von Frey filament stimulation in control animals, and 5-HT2AR−/− mice were reported to respond to von Frey filament stimulation exactly as wild-type animals (Kayser et al., 2007). Moreover, we have observed that the 5-HT2AR immunoreactivity is not modified in the ventral horn and intermediate grey of ddC-treated rats (not shown).

Results obtained in knock-out mice devoid of 5-HT2AR gene largely confirm those obtained in the rat. However, the time course of the development of neuropathy is difficult to compare between the two species because the doses and route of administration of the drug are different. Already four days after the first 500 mg/kg i.p. ddC injection, mice developed a mechanical hypersensitivity, and this phenomenon increased up to nine days after the treatment. Not only was this mechanical hypersensitivity almost totally absent in 5-HT2AR−/− mice but it was also largely attenuated in heterozygous 5-HT2AR+/− mice. We have verified using immunocytochemistry that 5-HT2AR immunolabelling was totally absent in 5-HT2AR−/− mice and intermediate in heterozygous mice. The intensity of neuropathy, reflected by mechanical hypersensitivity, is thus dependent on the expression of the 5-HT2AR. The changes associated with 5-HT2AR gene knock-out suggest that the receptor may also play a role in neuropathic pain at supraspinal sites. This may well be the case since 5-HT2AR are present in almost all regions involved in the control and transmission of nociceptive messages including the reticular formation, the nucleus raphe magnus, the central grey, the thalamus, cerebral cortex and limbic structures (Pompeiano et al., 1994; Jakab and Goldman-Rakic, 1998; Cornea-Hebert et al., 1999; Miner et al., 2003). Moreover, it has already been shown that 3 h after peripheral carrageenan inflammation, 5-HT2AR mRNA is up-regulated not only in the dorsal spinal cord, but also in the nucleus raphe magnus, the ventrolateral grey and the dorsal raphe nucleus (Zhang et al., 2001). A 5-HT2AR antagonist, sarpogrelate, used in Japan as an antiplatelet agent to treat patients with arteriosclerosis, significantly improves allodynia in neuropathic rats (Hashizume et al., 2007). Our data suggest that the site of action of 5-HT2AR antagonists may be not only at the periphery but also at the spinal and perhaps supraspinal levels. Accordingly, 5-HT2AR antagonists might help to alleviate various kinds of neuropathic pain.

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