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RESEARCH PAPER

A spinal mechanism of action for duloxetine in a rat model of painful diabetic neuropathy

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BACKGROUND AND PURPOSE
This study was designed to clarify mechanisms responsible for the anti-allodynic effects of duloxetine in diabetes.

EXPERIMENTAL APPROACH
The streptozotocin-induced diabetic rat model was used to compare the efficacy of duloxetine, 5-HT, the 5-HT_{2A} receptor agonist [1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI)] and two antagonists (ketanserin and pruvanserin) on tactile allodynia.

KEY RESULTS
Systemic or intrathecal injection of duloxetine alleviated tactile allodynia in diabetic rats. The effect of systemic duloxetine was reduced by intrathecal administration of ketanserin or pruvanserin, indicating participation of spinal 5-HT_{2A} receptors in the mechanism of action of duloxetine. In contrast to spinal delivery, systemic and local peripheral injections of ketanserin or pruvanserin alleviated tactile allodynia in diabetic rats. This effect was reversed immediately after systemic or local DOI injection.

CONCLUSIONS AND IMPLICATIONS
These results support the involvement of spinal 5-HT_{2A} receptors in the ability of duloxetine to ameliorate painful diabetic neuropathy. Our data also suggest that the role of 5-HT_{2A} receptors depends on the level of the neuraxis at which activation takes place, with peripheral activation contributing to tactile allodynia in diabetic rats, whereas spinal activation of this receptor alleviates tactile allodynia. The development of selective peripheral 5-HT_{2A} receptor antagonists may offer a novel approach for the treatment of diabetic neuropathic pain.

Abbreviations
AUC, area under the 50% PWT time curve; ECL, enhanced chemiluminescence; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride; DRG, dorsal root ganglia; i.t., intrathecal; PBS, phosphate buffered saline buffer; PWT, paw withdrawal threshold

Introduction

Diabetes mellitus is an increasingly common chronic medical condition, affecting over 100 million people worldwide. Up to 60% of diabetic patients develop some form of peripheral neuropathy and around 20% will develop neuropathic pain, including spontaneous pain, paresthesia, dysethesia, hyperalgesia and allodynia (Boulton *et al.*, 1983; Partanen *et al.*, 1995). The current first-line treatments for painful diabetic neuropathy include anticonvulsants, tricyclic antidepressants, opioids and serotonin (5-HT) and noradrenaline reuptake inhibitors (Ziegler, 2009). Duloxetine (Cymbalta) is a 5-HT-noradrenaline reuptake inhibitor used to treat depression that also alleviates allodynia in several inflammatory and neuropathic pain models (Iyengar *et al.*, 2004; Jones *et al.*, 2005; Joshi *et al.*, 2006; Kuhad *et al.*, 2009; Munro,

2009; Piesla *et al.*, 2009; Vranken *et al.*, 2011). Clinical studies have confirmed the efficacy of duloxetine against pain in diabetic patients (Goldstein *et al.*, 2005; Raskin *et al.*, 2005; Wernicke *et al.*, 2006), and the drug has been approved by the US Food and Drug Administration for management of painful diabetic neuropathy. The sites and mechanisms of action responsible for the pain-relieving effects of duloxetine in diabetes remain unclear.

Duloxetine inhibits transporters of 5-HT and noradrenaline, thereby increasing local levels of these neurotransmitters (Engleman *et al.*, 1995; Koch *et al.*, 2003) and promoting persistence of their actions. 5-HT exerts its action through binding to a range of receptors (Hoyer *et al.*, 1994), including the 5-HT_{2A} receptor (nomenclature follows Alexander *et al.*, 2009) which has been implicated in modulation of neuropathic or inflammatory pain (Obata *et al.*, 2000; 2001; Sasaki *et al.*, 2001; Okamoto *et al.*, 2002; Nitanda *et al.*, 2005; Honda *et al.*, 2006). Studies in animal models suggest that the nature of the involvement of the 5-HT_{2A} receptor may be location specific. Indeed, neuropathic pain is reported to be alleviated by both activation of spinal 5-HT_{2A} receptors and systemic administration of the 5-HT_{2A} receptor antagonist ketanserin (Obata *et al.*, 2001; Nitanda *et al.*, 2005; Honda *et al.*, 2006).

Streptozotocin-diabetic rats display evidence of sensory dysfunction, such as tactile allodynia and hyperalgesia to mechanical and thermal stimuli (Calcutt, 2004), supporting their use to investigate aetiological mechanisms of painful diabetic neuropathy and to screen for potential therapeutic agents (Calcutt and Backonja, 2007). A recent study has reported that inhibitors of 5-HT and noradrenaline reuptake can modulate diabetes-induced allodynia via a spinal mechanism of action (Ikeda *et al.*, 2009). In the present study, we have used the streptozotocin-diabetic rat model to investigate the specific site of action of the anti-neuropathic pain properties of duloxetine and the potential role of the 5-HT_{2A} receptor in its mechanism of action.

Methods

Animals

All animal care and experimental studies were carried out according to the protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Animals were housed by two to three per cage, with free access to food and water and were maintained under conditions approved by the American Association for the Accreditation of Laboratory Animal Care. All experiments were performed using 220–250 g adult female Sprague–Dawley rats (Harlan Industries, San Diego CA, USA). Female rats were used to provide consistency with our previous studies of diabetes-induced allodynia, and we have found no sex differences in the effects of diabetes on nocifensive behaviour (Malmberg *et al.*, 1993; Calcutt *et al.*, 1996).

Induction of diabetes

Insulin-deficient diabetes was induced following an overnight fast by a single intraperitoneal (i.p.) injection of streptozotocin (Sigma, St. Louis, MO, USA), 50 mg·kg^{-1} dissolved in 0.9% sterile saline. Hyperglycaemia was confirmed using a strip-operated reflectance meter (One Touch Ultra, Life Scan Inc., Milpitas, CA, USA) in a blood sample obtained by tail prick 4 days after streptozotocin injection. The day of the experiment (between 6 and 10 weeks of diabetes), blood glucose levels were again determined and only rats with blood glucose levels $\geq$15 mmol·L^{-1} were included in the study.

Assessment of allodynia

Tactile allodynia was determined by measuring hind paw withdrawal in response to probing with a series of calibrated filaments (von Frey filaments, Stoelting, Wood Dale, IL, USA). The force applied ranged from 0.4 to 15 g. Tactile allodynia was assessed hourly for 5–6 h with an additional 30 min time point during the first hour. The 50% paw withdrawal threshold (50% PWT) was determined by using the up : down method (Chaplan *et al.*, 1994).

Stepping and righting behaviour

Rats in all groups were observed for behavioural or motor function changes induced by the treatments. This was assessed, but not quantified, by testing the animals' ability to stand and walk in a normal posture, as proposed elsewhere (Chen and Pan, 2001).

Intrathecal (i.t.) catheterization

At 7–8 weeks of diabetes, rats were implanted with an i.t. PE-10 catheter under isoflurane anaesthesia (Yaksh and Rudy, 1976). Briefly, rats were placed in a stereotaxic head holder, and the cisternal membrane exposed. The membrane was pierced, and a PE-10 catheter (8.5 cm) passed intrathecally to the level of the lumbar enlargement. Rats were allowed to recover from surgery for at least 5 days before use. Animals showing any signs of motor impairment were killed humanely.

Western blotting

Mid-thigh sciatic nerve, L4-L5 dorsal root ganglia (DRG) and lumbar spinal cord were collected after decapitation of anaesthetized rats, homogenized in ice-cold phosphate buffered saline buffer and antiprotease, and centrifuged (14 000× *g* for 30 min at 4°C). Supernatant was collected and pellet was homogenized again with Chaps buffer (50 mM Tris HCl, pH 7.6, 10 mM Chaps, 0.05 mM EDTA-disodium, protease inhibitor cocktail) and centrifuged (14 000 × *g* for 30 min at 4°C). Samples of total protein (10 µg) were separated on 4–12% SDS-PAGE Bis–Tris gels (Novex, Invitrogen, Carlsbad, CA, USA) and immunoblotted on nitrocellulose membrane. Membranes were incubated with anti-5-HT_{2A} receptor (1/500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-β-actin antibodies (1/5000, Sigma), followed by incubation with horseradish peroxidase-linked anti-rabbit secondary antibody (1/20 000, Santa Cruz Biotechnology) or anti-mouse secondary antibody (1/20 000, Santa Cruz Biotechnology). Blots were developed using an Enhanced Chemiluminescence Western-blotting protocol (Lumilight Roche Applied Science, Indianapolis, IN, USA). To detect on the same blot, 5-HT_{2A} receptor and actin proteins that have similar molecular weights (55 vs. 43 kDa, respectively), previously anti-5-HT_{2A} receptor bound antibodies were removed with stripping buffer (Pierce, Rockford, Il, USA) before incubation with anti-

actin antibodies. Quantification of immunoreactivity was performed by densitometric scanning using Quantity One software (Bio-Rad, San Diego, CA, USA). For each animal, band intensities were normalized by calculating the ratio of the intensity of bands corresponding to 5-HT_{2A} receptor protein to the intensity of the band corresponding to β-actin. The β-actin normalized data for each lane were expressed as a percentage of the group mean of values obtained from all control rats run on the same gel.

Administration of drugs

For local drug administration, rats received a subcutaneous (s.c.) injection (50 µL) into the dorsal surface of the hind paw of vehicle, duloxetine, DOI, ketanserin, pruvanserin or cromolyn. For systemic drug administration, drug was given in a volume of 1 mL·kg^{-1} for ketanserin (i.p.), pruvanserin (i.p.) or DOI (s.c.), or 2 mL·kg^{-1} for duloxetine (i.p.). For i.t. administration, drugs were delivered in a volume of 10 µL followed by 10 µL saline. Vehicle groups received sterile water for duloxetine; saline for DOI, ketanserin or cromolyn; sterile water containing Tween 80 for pruvanserin. PWTs were determined before and 30, 60, 120, 180, 240 and 300 min after drug administration for i.t. and local-peripheral treatments; an additional hour was recorded for systemic treatments. Doses and drug administration schedules for duloxetine, DOI, ketanserin, pruvanserin and cromolyn were based on previous reports (Koch *et al.*, 2003; Adamec *et al.*, 2004; Dogrul and Seyrek, 2006; Mbaki and Ramage, 2008) or our own pilot experiments.

As the aim of this study was to demonstrate that the anti-allodynic mechanism of duloxetine was mediated by 5-HT_{2A} receptors, our schedule of administration was chosen in order to block activated 5-HT_{2A} receptors and when the anti-allodynic effect could be reduced, rather than prevented. The time of injection of antagonists or agonists was determined by the time of peak effect of duloxetine, DOI, ketanserin or pruvanserin, depending on the route of administration. To determine the effect of duloxetine on tactile allodynia in diabetic rats, duloxetine was administered by different routes so that rats received vehicle (sterile water) or duloxetine i.p. (20 mg·kg^{-1}), i.t. (20 µg) or locally-peripherally (50 µg). The 5-HT_{2A} receptor antagonists ketanserin (20 µg) or pruvanserin (20 µg) were injected i.t., 210 min after systemic administration of duloxetine (20 mg·kg^{-1} i.p.) but these antagonists were given i.t. 90 min after duloxetine (20 µg) was administered i.t. To investigate the contribution of 5-HT_{2A} receptors to diabetes-induced allodynia more specifically, DOI was delivered i.t. (20 µg) to rats, alone or followed by i.t. injection of either 20 µg pruvanserin or 20 µg ketanserin, 40 min after DOI. Ketanserin (1 mg·kg^{-1}) or pruvanserin (10 mg·kg^{-1}) were delivered i.p., alone or followed by 0.1 mg·kg^{-1} s.c. DOI, 150 min after ketanserin or pruvanserin. Similarly, rats received s.c. injection of ketanserin (50 µg) or pruvanserin (50 µg) into the dorsal surface of the hind paw, alone or followed by DOI (50 µg) 90 min later. To determine if ketanserin or pruvanserin acted locally, they were administered to the right paw and PWT measured in both ipsilateral and contralateral paws. Finally, cromolyn, a mast cell membrane stabilizer, was given locally at a dose of 800 ng per·paw.

Data analysis

All experimental results are given as median for non-parametric data (upper and lower cut-offs for tactile allodynia) or mean ± SEM [for area under the 50% PWT time curve (AUC)] with 4 to 15 animals per group. The mean of the 50% PWTs obtained with the von Frey filaments were used to construct PWT curves as a function of time for each group. AUC was calculated for a period of 5–6 h by the trapezoidal method, as previously described by Tallarida and Murray (1981) and calculated as: AUC = [(PWT at time 1 + PWT at time 2)/2] × (time 2–time 1) + [(PWT at time 2 + PWT at time 3)/2] × (time 3–time 2). . . . One–way ANOVA, followed by Tukey's test or *t*-tests were used to compare differences between treatments. Differences were considered to reach statistical significance when $P < 0.05$.

Materials

Streptozotocin, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI, 5-$HT_{2A/2C}$ receptor agonist) and ketanserin (5-$HT_{2A/1D}$ receptor antagonist) were purchased from Sigma. Pruvanserin [EMD 281014, selective 5-HT_{2A} receptor antagonist (Bartoszyk *et al.*, 2003)] was provided by Dr Gerd Bartoszyk (Merck KGaA, Darmstadt, Germany). The prescription formulation of Cymbalta (Eli Lilly, Indianapolis, IN, USA) was used as a source of duloxetine hydrochloride. Cromolyn sulphate was obtained from MP Biomedicals LLC (Solon, OH, USA). Streptozotocin, DOI, ketanserin and cromolyn were dissolved in 0.9% sterile saline. Duloxetine was dissolved in sterile water and pruvanserin was dissolved in sterile water containing Tween 80.

Results

Anti-allodynic effect of duloxetine

Streptozotocin injection resulted in hyperglycaemia within 3–4 days. The blood glucose concentration of the cohort of streptozotocin-injected rats was significantly ($P < 0.001$) increased (29.7 ± 0.9 mmol·L^{-1}) compared with control rats (5.7 ± 0.2 mmol·L^{-1}).

Studies were performed 6–10 weeks after induction of diabetes as the degree of allodynia was stable over this period. Tactile allodynia, defined as a 50% PWT < 5 g, was consistently present in streptozotocin-injected rats compared with non-diabetic rats (group median PWT = 15 g) and was significantly ($P < 0.001$) alleviated by systemic administration of 20 mg·kg^{-1} duloxetine (Figure 1). The maximal anti-allodynic effect was reached 4 h after administration, and efficacy persisted over the next 2 h (Figure 1A). Administration of duloxetine to non-diabetic rats did not affect PWT. Vehicle (sterile water) administration did not affect PWT and no changes in behavioural or motor functions, as assessed by the stepping and righting reflexes, were observed in either group at the doses employed.

Duloxetine was also delivered to diabetic rats by local paw injection or directly to the spinal cord to clarify potential sites of action. Intrathecal delivery of 20 µg duloxetine alleviated tactile allodynia in diabetic rats, with the maximal effect reached at 2 h (Figure 1A). Local administration of duloxetine to the paw (50 µg per paw) did not alter tactile allodynia in

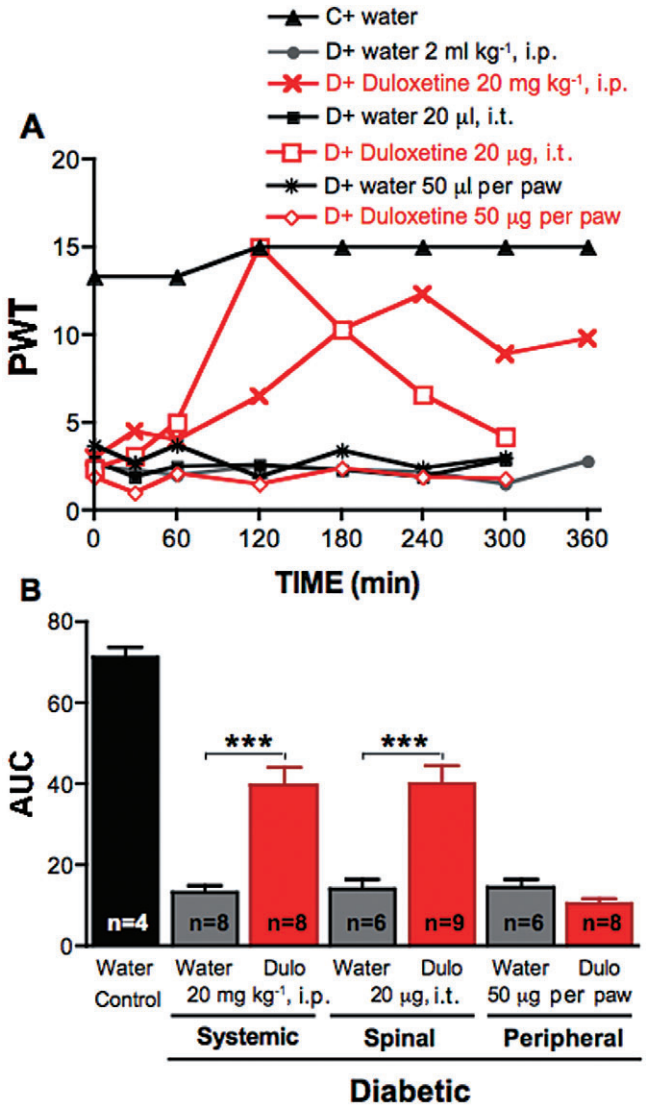

Figure 1

Effect of duloxetine on tactile allodynia in diabetic rats. (A) Tactile responses, represented as 50% paw withdrawal threshold (PWT), for control rats (C) receiving i.p. injection of water, for diabetic rats (D) receiving i.p. injection of water or duloxetine at 20 mg·kg^{-1}, intrathecal (i.t.) injection of water or duloxetine (20 µg), or local injection in the paw of water or duloxetine (50 µg). Data are expressed as median, n = 4–9 per·group as indicated on bar. (B) Area under the 50% PWT curve (AUC) from 0 to 300 min for control rats receiving water, diabetic rats receiving water or duloxetine (Dulo) by systemic, spinal or peripheral route as indicated on graph. Data are expressed as mean + SEM, ***P < 0.001 using unpaired Student's t-test against vehicle treated animals for each route of administration.

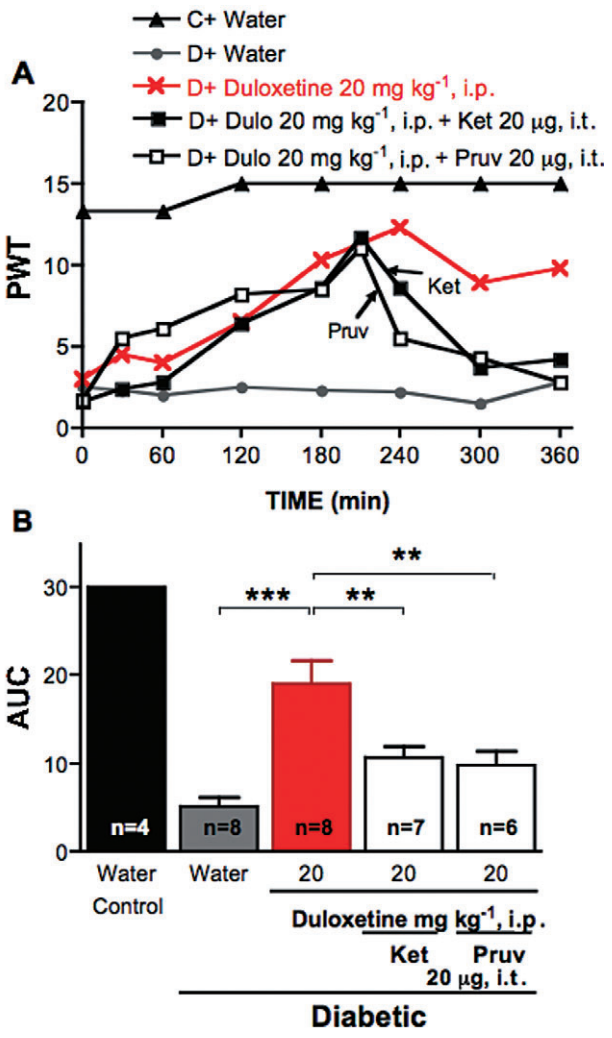

Figure 2

Effect of ketanserin or pruvanserin on the anti-allodynic effects of systemic duloxetine. (A) Tactile responses, represented as 50% paw withdrawal threshold (PWT), for control rats (C) receiving water and for diabetic rats (D) receiving i.p. injection of water, duloxetine at 20 mg·kg^{-1} or duloxetine (Dulo) followed 210 min later by i.t. injection of ketanserin (Ket, 20 µg) or pruvanserin (Pruv, 20 µg). Arrows indicate the time of injection of ketanserin (Ket) or pruvanserin (Pruv). Data are expressed as median, n = 4–8 per group as indicated on bar. (B) Area under the 50% PWT curve (AUC) from 240 to 360 min for control rats receiving water, diabetic rats receiving i.p. injection of water, duloxetine alone or duloxetine followed by i.t. injection of ketanserin (Ket) or pruvanserin (Pruv) as indicated on graph. Data are expressed as mean + SEM. **P < 0.01, ***P < 0.001 using one-way ANOVA followed by Tukey's *post hoc* test.

diabetic rats (Figure 1), although a slight decrease of PWT was noticeable in the first hour after duloxetine administration, but did prompt flinching of the injected paw, implying pronociceptive properties. To determine how systemic duloxetine might exert its spinal anti-nociceptive properties, duloxetine (20 mg·kg^{-1}) was given i.p., followed 210 min later by an i.t. injection of 20 µg ketanserin, a 5-HT$_{2A/1D}$ receptor antagonist. Similar experiments were performed with a selective 5-HT$_{2A}$ receptor antagonist, pruvanserin (Bartoszyk *et al.*, 2003). The alleviation of allodynia induced by systemic duloxetine was reversed by i.t. ketanserin or pruvanserin, indicating participation of spinal 5-HT$_{2A}$ receptors in the mechanism of action of duloxetine (Figure 2). Similarly, the antiallodynic effect after i.t. administration of duloxetine was reversed by i.t. ketanserin or pruvanserin administration (Figure 3).

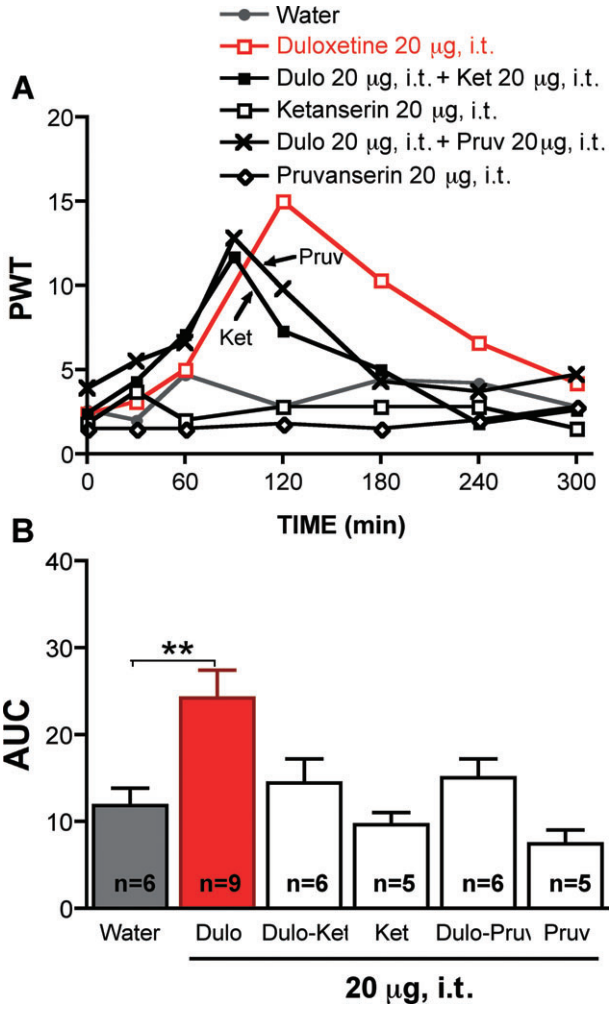

Figure 3

Effect of ketanserin or pruvanserin on the anti-allodynic effects of duloxetine given i.t.. (A) Tactile responses, represented as 50% paw withdrawal threshold (PWT) for diabetic rats receiving i.t. injection of water, duloxetine (20 μg), ketanserin (20 μg), pruvanserin (20 μg) or duloxetine followed 90 min later by i.t. injection of ketanserin (Ket, 20 μg) or pruvanserin (Pruv, 20 μg). Arrows indicate the time of injection of ketanserin (Ket) or pruvanserin (Pruv). Data are expressed as median, n = 5–9 per group as indicated on bar. (B) Area under the 50% PWT curve (AUC) from 90 to 300 min for diabetic rats receiving i.t. injection of water, duloxetine alone (Dulo) or duloxetine followed by i.t. injection of ketanserin (Ket) or pruvanserin (Pruv) as indicated on graph. Data are expressed as mean + SEM. ** $P < 0.01$ using one-way ANOVA followed by Tukey's *post hoc* test.

Effects of 5-HT$_{2A}$ receptor agonist and antagonist in diabetic rats

Intrathecal injection of the 5-HT$_{2A/2C}$ receptor agonist DOI (20 μg) induced a rapid alleviation of tactile allodynia in diabetic rats, with peak efficacy occurring 1–2 h after injection (Figure 4A). Neither pruvanserin nor ketanserin nor vehicle (saline) alone had any effect (Figure 4). The anti-allodynic effect of DOI was reversed by subsequent i.t. administration of ketanserin (20 μg) or pruvanserin (20 μg), given 40 min after DOI. These results suggest that 5-HT$_{2A}$ receptor activation is anti-allodynic at the level of the spinal cord.

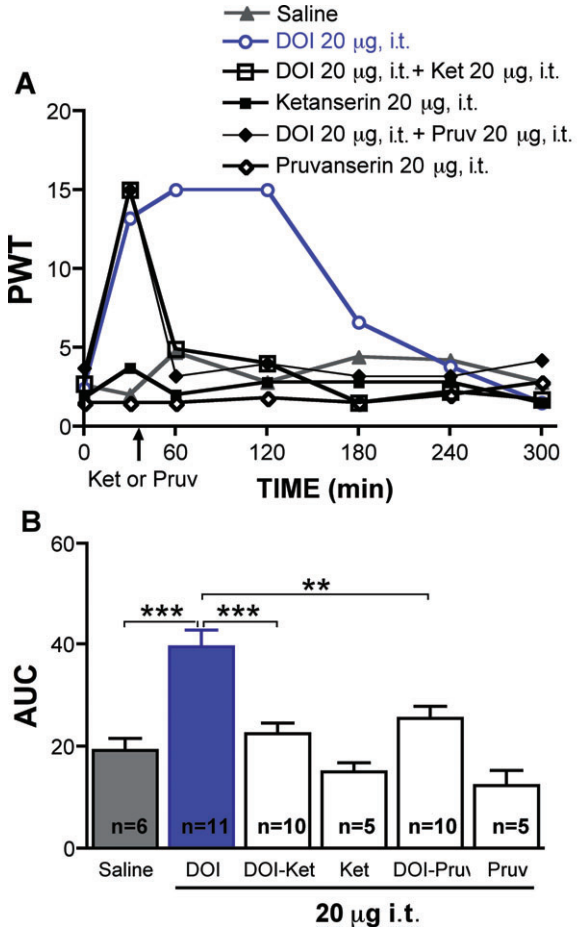

Figure 4

Effect of spinal delivery of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), ketanserin or pruvanserin on tactile allodynia in diabetic rats. (A) Tactile responses, represented as 50% paw withdrawal threshold (PWT), for diabetic rats receiving intrathecal injection of saline, DOI (20 μg), ketanserin (20 μg), pruvanserin (20 μg) or DOI followed 40 min later by i.t. injection of ketanserin (Ket, 20 μg), or pruvanserin (Pruv, 20 μg). Data are expressed as median, n = 5–11 per group as indicated on bar. (B) Area under the 50% PWT curve (AUC) from 0 to 300 min for diabetic rats receiving i.t. injection of saline or 5-HT$_{2A}$ agonist and/or antagonist, as indicated on graph. Data are expressed as mean + SEM. ** $P < 0.01$, *** $P < 0.001$ using one-way ANOVA followed by Tukey's *post hoc* test.

Systemic (1 or 10 mg·kg^{-1} i.p.) and local (50 μg per paw) injection of the 5-HT$_{2A}$ receptor antagonists ketanserin or pruvanserin significantly alleviated tactile allodynia in streptozotocin-diabetic rats ($P < 0.001$, Figures 5 and 6). The systemic and local anti-allodynic effects of ketanserin or pruvanserin were reversed immediately after systemic (0.1 mg·kg^{-1} s.c.) or local (50 μg per paw) injection of DOI (Figures 5 and 6). Systemic (0.1 mg·kg^{-1} s.c.) or local (50 μg per paw) administration of DOI did not modify tactile responses in either control (data not shown) or diabetic rats (Figures 5 and 6), although a slight decrease of PWT was noticeable for the first hour after DOI administration in the paw of diabetic rats. These results indicate a pro-allodynic

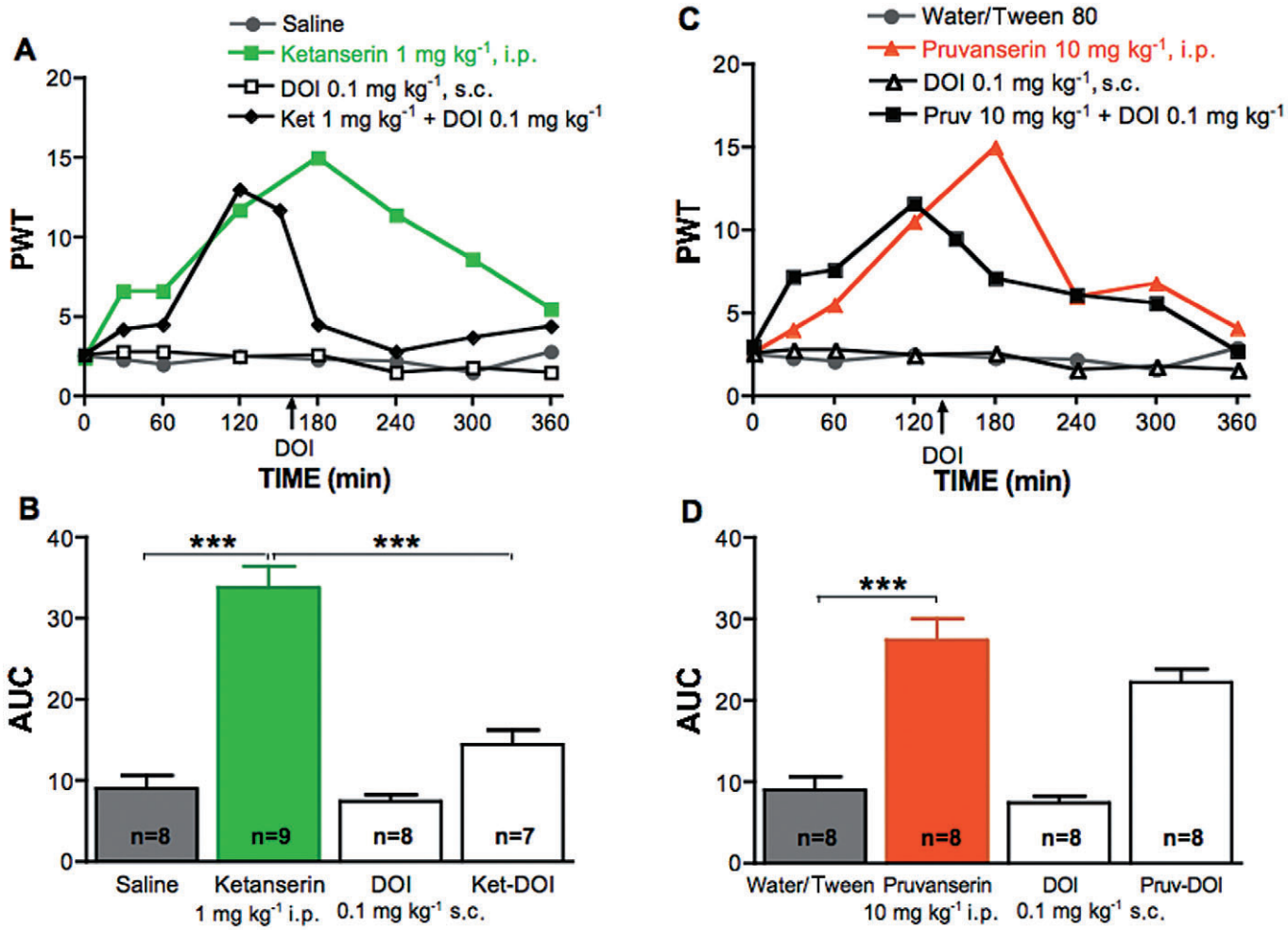

Figure 5

Effect of systemic delivery of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), ketanserin or pruvanserin on tactile allodynia in diabetic rats. (A) Tactile responses, represented as 50% paw withdrawal threshold (PWT), for diabetic rats receiving i.p. injection of saline, ketanserin (1 mg·kg^{-1}), s.c. injection of DOI (0.1 mg·kg^{-1}) or i.p. injection of ketanserin (Ket, 1 mg·kg^{-1}) followed 150 min later by subcutaneous injection of DOI (0.1 mg·kg^{-1}). Data are expressed as median, n = 7–9 per group as indicated on bar. (B) Area under the 50% PWT curve (AUC) from 150 to 360 min for diabetic rats receiving systemic injection of saline, ketanserin alone, with DOI or DOI alone, as indicated on graph. Data are expressed as mean + SEM. ***P < 0.001 using one-way ANOVA followed by Tukey's *post hoc* test. (C) Tactile responses, represented as 50% paw withdrawal threshold (PWT), for diabetic rats receiving i.p. injection of water containing Tween80, pruvanserin (10 mg·kg^{-1}), s.c. injection of DOI (0.1 mg·kg^{-1}) or i.p. injection of pruvanserin (Pruv, 10 mg·kg^{-1}) followed 150 min later by subcutaneous injection of DOI (0.1 mg·kg^{-1}). Data are expressed as median, n = 7–9 per group as indicated on bar. (D) Area under the 50% PWT curve (AUC) from 150 to 360 min for diabetic rats receiving systemic injection of water containing Tween 80 or pruvanserin alone, followed by DOI or DOI alone, as indicated on graph. Data are expressed as mean + SEM. ***P < 0.001 using one-way ANOVA followed by Tukey's *post hoc* test.

role of 5-HT$_{2A}$ receptor activation in the periphery in diabetic rats. Local administration of the mast cell stabilizer cromolyn (800 µg per paw) did not inhibit allodynia in diabetic rats (Figure 6A, B).

5-HT$_{2A}$ receptor protein expression in diabetic rat tissues

Quantification of 5-HT$_{2A}$ receptor protein levels by Western blot indicated no change in spinal cord, DRG or sciatic nerve of diabetic rats compared with controls (Figure 7).

Discussion

Duloxetine is a potent and selective 5-HT and noradrenaline reuptake inhibitor with little or no affinity for 5-HT, noradrenaline, dopamine, acetylcholine or opioid receptors (Bymaster *et al.*, 2001). It is widely used as an antidepressant with a mechanism of action based on its ability to inhibit 5-HT and noradrenaline transporters (Koch *et al.*, 2003) and therefore, to increase extracellular monoamine levels in the brain (Engleman *et al.*, 1995; Koch *et al.*, 2003). Duloxetine is also one of the first prescription drugs approved by the US

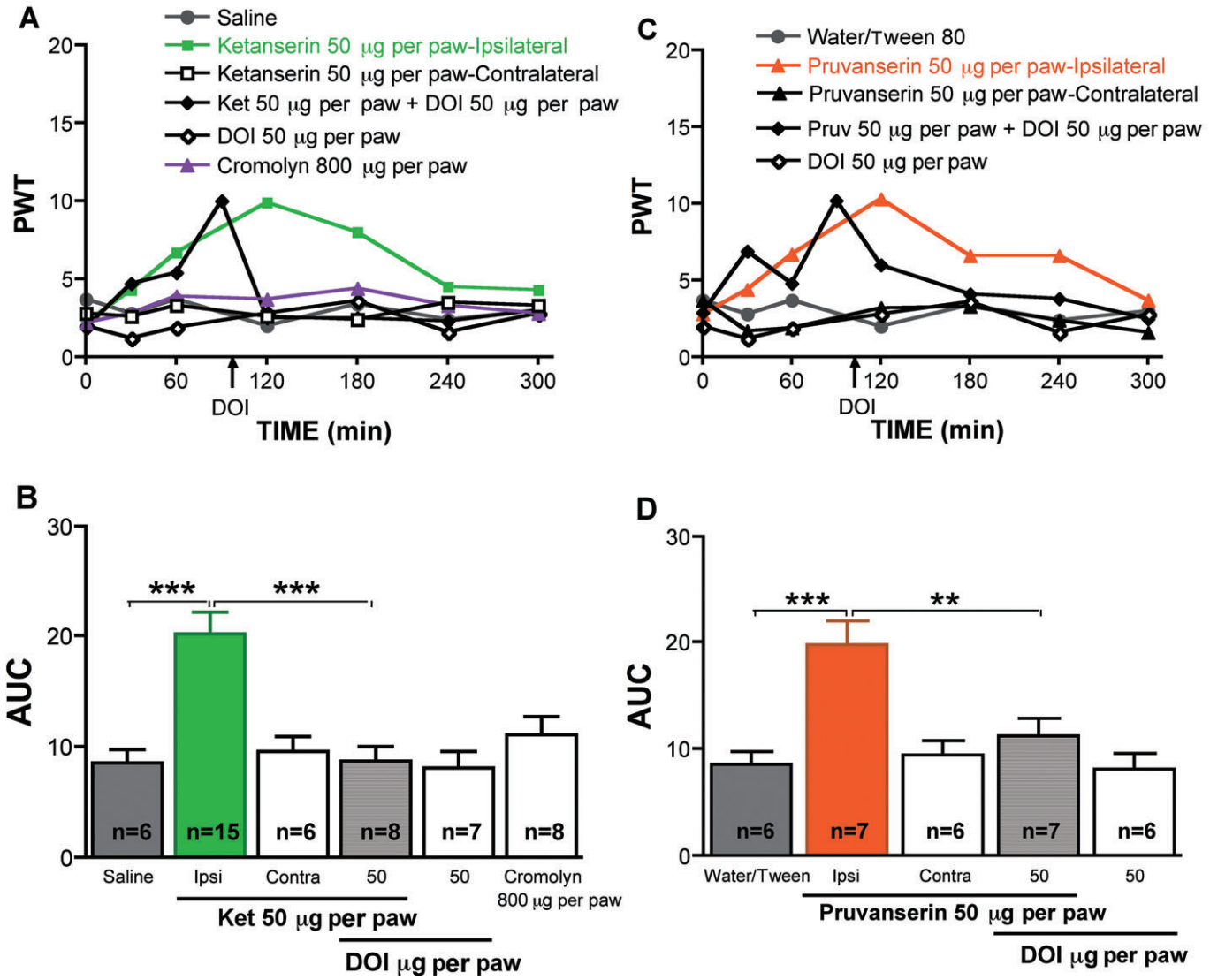

Figure 6

Effect of local peripheral delivery of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), ketanserin or pruvanserin on tactile allodynia in diabetic rats. (A) Tactile responses, represented as 50% paw withdrawal threshold (PWT), for diabetic rats receiving local injection in the paw of saline, ketanserin (50 μg, ipsilateral), ketanserin (contralateral), DOI (50 μg, ipsilateral), cromolyn (800 μg), or ketanserin (Ket) followed 90 min later by paw injection of DOI (50 μg). Data are expressed as median, n = 6–15 per group as indicated on bar. (B) Area under the 50% PWT curve (AUC) from 120 to 300 min for diabetic rats receiving injections in the paw of saline, 5-HT_{2A} antagonist ipsilaterally, contralaterally, or followed by paw injection of DOI and for diabetic rats receiving DOI alone or cromolyn alone, as indicated on graph. Data are expressed as mean + SEM. ***P < 0.001 using one-way ANOVA followed by Tukey's *post hoc* test. (C) Tactile responses, represented as 50% paw withdrawal threshold (PWT), for diabetic rats receiving local injection in the paw of water containing Tween 80, pruvanserin (50 μg, ipsilateral), pruvanserin (contralateral), DOI (50 μg, ipsilateral), or pruvanserin (Pruv, followed 90 min later by paw injection of DOI (50 μg,). Data are expressed as median, n = 6–7 per group as indicated on bar. (B) Area under the 50% PWT curve (AUC) from 120 to 300 min for diabetic rats receiving injection in the paw of water containing Tween 80, 5-HT_{2A} antagonist ipsilaterally, contralaterally, followed by paw injection of DOI or DOI alone, as indicated on graph. Data are expressed as mean + SEM. **P < 0.01, ***P < 0.001 using one-way ANOVA followed by Tukey's *post hoc* test.

Food and Drug Administration for the management of the pain associated with diabetic neuropathy (Lunn *et al.*, 2009). The mechanism of action by which duloxetine alleviates pain is considered to be distinct from its actions as an antidepressant (Perahia *et al.*, 2006). In patients suffering from pain and major depressive disorder, duloxetine treatment resulted in significant improvement of pain symptoms without changes in depressive symptoms, suggesting different mechanisms for relief of pain and depression (Brannan *et al.*, 2005). In addition, duloxetine showed efficacy in diabetic patients suffering from peripheral painful neuropathy without depression (Goldstein *et al.*, 2005; Raskin *et al.*, 2005). The precise mechanism of pain relief is not clear, although the enhanced involvement of 5-HT and noradrenaline in descending inhibitory pathways or direct action on neuronal Na^+ currents have been evoked as plausible

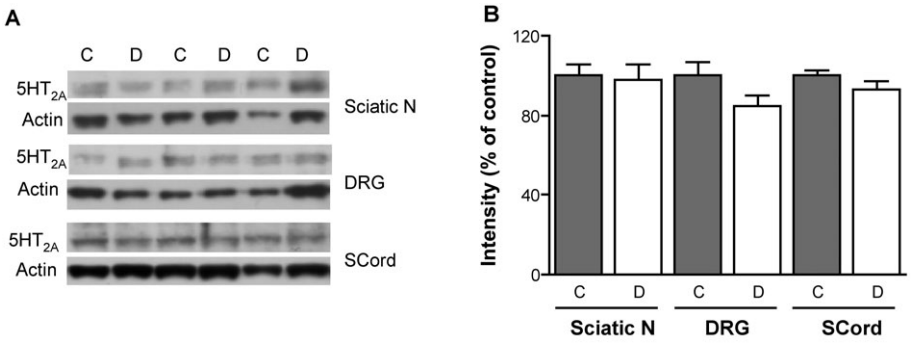

Figure 7

Western blot analysis of 5-HT_{2A} receptor protein levels in rat sciatic nerve, dorsal root ganglia and spinal cord. (A) Representative Western blots and (B) densitometric quantification for 5-HT_{2A} receptor protein (55 kDa) and β-actin (43 kDa) in the sciatic nerve (Sciatic N), dorsal root ganglia (DRG) and spinal cord (SCord) of control (C) and streptozotocin-diabetic (D) rats. Data are expressed as mean + SEM, n = 5–7 per group.

mechanisms (Millan, 2002; Thor *et al.*, 2007; Wang *et al.*, 2010).

Several studies have shown that systemic duloxetine is anti-nociceptive in models of traumatic and inflammatory pain (Iyengar *et al.*, 2004; Bomholt *et al.*, 2005; Jones *et al.*, 2005; Joshi *et al.*, 2006; Munro, 2009; Piesla *et al.*, 2009), but efficacy of duloxetine in models of painful diabetic neuropathy has been restricted to a recent report of the alleviation of thermal hyperalgesia in mice (Kuhad *et al.*, 2009). In the present study, we have shown that systemic and spinal, but not peripheral, administration of duloxetine alleviated tactile allodynia in diabetic rats, in the absence of effects on motor function. Maximal efficacy was similar when duloxetine was delivered either systemically or spinally, with the latter showing a predictably faster onset. Duloxetine may therefore replenish depleted 5-HT levels in the CNS of diabetic rats (Sandrini *et al.*, 1997). Our results are consistent with the alleviation of mechanical hyperalgesia after i.t. injection of 5-HT in diabetic rats (Bardin *et al.*, 2000), the attenuation of thermal hyperalgesia in diabetic mice by the 5-HT reuptake inhibitor fluoxetine (Anjaneyulu and Chopra, 2004) and a report which showed that other 5-HT reuptake inhibitors, selective or non-selective, showed anti-allodynic effects in diabetic rats after spinal delivery (Ikeda *et al.*, 2009). The ability of spinally delivered ketanserin or pruvanserin, a selective 5-HT_{2A} receptor antagonist, to block the anti-allodynic effects of duloxetine also indicates that the mechanism of action by which systemic duloxetine alleviated painful diabetic neuropathy included enhancement of 5-hydroxytryptaminergic inhibitory systems operating through spinal 5-HT_{2A} receptors. Involvement of spinal 5-HT_{2A} receptor activation in alleviation of neuropathic pain has been demonstrated in nerve injury models (Obata *et al.*, 2001; Honda *et al.*, 2006) and in formalin-evoked hyperalgesia (Sasaki *et al.*, 2001). Alleviation of allodynia after spinal delivery of 5-HT_{2A} agonist suggests possible impairment of the spinal 5-HT_{2A} receptor that could contribute to spinal disinhibition and subsequent pain-associated behaviours, as it does in other models of neuropathic pain (Dubner and Ren, 1999). However, 5-HT_{2A} receptor protein levels were unchanged in spinal cord from diabetic rats. Similarly, no changes were observed in the peripheral nervous system.

Although enhanced stimulation of spinal 5-HT_{2A} receptors alleviated allodynia in diabetic rats, we also found that the effects of 5-HT_{2A} receptor stimulation and blockade were reversed when drugs were administered in the periphery. Indeed, peripheral and systemic administration of ketanserin or pruvanserin alleviated tactile allodynia in diabetic rats, and in both paradigms the effect was reversed by DOI. In addition, direct injection of duloxetine or DOI into the paw of diabetic rats produced a slight decrease of PWT in the first hour after administration. Our data implicate activation of peripheral 5-HT_{2A} receptors in the allodynia of diabetic rats and are in agreement with the reported excitatory role of the peripheral 5-HT_{2A} receptor in other pain modality models such as neuropathic or inflammatory pain models (Obata *et al.*, 2000; Sasaki *et al.*, 2001; Okamoto *et al.*, 2002; Nitanda *et al.*, 2005). The peripheral sources of 5-HT include mast cells, platelets and endothelial cells (Dray, 1995; Millan, 1999), while 5-HT_{2A} receptors are located on endothelial cells (Martin, 1994), Schwann cells (Yoder *et al.*, 1997; Gaietta *et al.*, 2003), the perikarya of sensory neurons in the DRG (Okamoto *et al.*, 2002; Van Steenwinckel *et al.*, 2009), and in axons of both myelinated and unmyelinated fibres (Carlton and Coggeshall, 1997; Okamoto *et al.*, 2002). The release of 5-HT from mast cells is specific to rodents (Sjoerdsma *et al.*, 1957; Benditt *et al.*, 1963), and we have excluded these cells as a source of the diabetes-induced, 5-HT-mediated allodynia by using the mast cell stabilizer cromolyn. Endothelial cells are another potential source of 5-HT, as they are perturbed by diabetes (Cameron *et al.*, 2001) and may release 5-HT into the endoneurial environment. Platelets abnormalities that occur in diabetes (Glassman, 1993) may also contribute to the peripheral pathogenesis of diabetic painful neuropathy via increased 5-HT release (Pietraszek *et al.*, 1992; Malyszko *et al.*, 1994). Alternatively, a number of cell types in the skin also produce 5-HT (Nordlind *et al.*, 2008). Investigating the location and mechanisms by which a peripheral 5-HT_{2A} receptor-mediated process contributes to allodynia in diabetic rats awaits future studies.

Duloxetine is a balanced 5-HT and noradrenaline reuptake inhibitor (Bymaster *et al.*, 2001), therefore, activation of the descending noradrenergic pathway may also play a role in the anti-allodynic effects of duloxetine. Our data

showed that, in our rat model, duloxetine acts predominantly via activation of 5-HT_{2A} receptors. Indeed, the effect of the non-selective 5-HT_{2A} receptor antagonist, ketanserin, parallels that of the selective antagonist, pruvanserin and their effects were reversed by DOI, a 5-$HT_{2A/2C}$ agonist. An additional support for the minor role of noradrenaline, but a major role of 5-HT and 5-HT_{2A} receptors in both diabetic painful neuropathy and the mechanism of action of duloxetine, is the rapid and complete reversal of the anti-allodynic effect by the 5-HT_{2A} receptor antagonists. In contrast, in normal rats, duloxetine suppressed spinal hyperactivity triggered by prostaglandin E_2, and that effect was blocked by a combination of 5-$HT_{1B/1D}$ and α_2-adrenoceptor antagonists (Tsukamoto *et al.*, 2010), implying a role for these receptors in the antinociceptive effect of duloxetine. Although we cannot totally exclude noradrenaline or other possible mechanisms, such as blockade of Na^+ currents (Wang *et al.*, 2010), 5-$HT_{1B/1D}$ and α_2-adrenoceptors (Tsukamoto *et al.*, 2010), from playing a role in the anti-allodynic effect of duloxetine, our data suggest that duloxetine alleviated allodynia in diabetic rat model mainly via the indirect activation of 5-HT_{2A} receptors in the spinal cord.

In summary, we have identified spinal 5-HT_{2A} receptors as a transduction system for the anti-allodynic and anti-hyperalgesic properties of duloxetine in diabetic rats. Our results also indicate that the role of the 5-HT_{2A} receptor in diabetes-induced allodynia and hyperalgesia depends on the level of the neuroaxis, with peripheral activation of the 5-HT_{2A} receptor contributing to tactile allodynia, whereas spinal activation of the receptor alleviates tactile allodynia. This is consistent with the known properties of 5-HT, whereby it can exert either algesic or analgesic effects depending on the site of action and the receptor subtype activated (Eide and Hole, 1993). These findings may also explain why, in contrast to acute effects in our rat study, patients with painful diabetic neuropathy noticed improvement of pain after at least 1 week of daily duloxetine administration (Goldstein *et al.*, 2005). Patients receive duloxetine via the oral route, limiting the amount of drug gaining access to the spinal cord, while in our rat study, duloxetine was injected systemically or directly to the spinal cord, bypassing the metabolism that follows oral administration and also the location-dependent actions of 5-HT. The combination of a 5-HT_{2A} receptor-mediated peripheral pro-allodynic effect with depletion of central 5-HT (Sandrini *et al.*, 1997; Padayatti and Paulose, 1999) and consequent impairment of 5-HT_{2A} receptor-mediated spinal inhibition may together promote pain states during diabetes. Peripherally restricted, selective 5-HT_{2A} receptor antagonists could offer a novel approach for the treatment of diabetic neuropathic pain.

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Conflict of interest

The authors state no conflict of interest.

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