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RESEARCH PAPER

5-HT$_{1A}$ receptors are involved in the effects of xaliproden on G-protein activation, neurotransmitter release and nociception

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Background and purpose: Xaliproden (SR57746A) is a 5-HT$_{1A}$ receptor agonist and neurotrophic agent that reduces oxaliplatin-mediated neuropathy in clinical trials. The present study investigated its profile on *in vitro* transduction, neurochemical responses and acute nociceptive pain tests in rats.
Experimental approach: Xaliproden was tested on models associated with 5-HT$_{1A}$ receptor activation including G-protein activation, extracellular dopamine and 5-HT levels measured by microdialysis and formalin-induced pain. Activation of 5-HT$_{1A}$ receptors was confirmed by antagonism with WAY100635.
Key results: Xaliproden exhibited high affinity for rat (r) and human (h) 5-HT$_{1A}$ receptors (pK_i = 8.84 and 9.00). In [^{35}S]GTPγS (guanosine 5′-O-(3-[^{35}S]thio)triphosphate) assays it activated both hippocampal r5-HT$_{1A}$ [pEC_{50}/E_{MAX} of 7.58/61% (%5-HT)] and recombinant h5-HT$_{1A}$ receptors (glioma C6-h5-HT$_{1A}$: 7.39/62%; HeLa-h5-HT$_{1A}$: 7.24/93%). In functional [^{35}S]GTPγS autoradiography, xaliproden induced labelling in structures enriched with 5-HT$_{1A}$ receptors (hippocampus, lateral septum, prefrontal and entorhinal cortices). Xaliproden inhibited *in vivo* binding of [^{3}H]WAY100635 to 5-HT$_{1A}$ receptors in mouse frontal cortex and hippocampus (ID_{50}: 3.5 and 3.3 mg·kg^{-1}, p.o. respectively). In rat, it increased extracellular dopamine levels in frontal cortex and reduced hippocampal 5-HT levels (ED_{50}: 1.2 and 0.7 mg·kg^{-1}, i.p. respectively). In a rat pain model, xaliproden inhibited paw licking and elevation (ED_{50}: 1 and 3 mg·kg^{-1}, i.p. respectively) following formalin injection in the paw. All effects were reversed by pretreatment with WAY100635.
Conclusions and implications: These results indicate that activation of 5-HT$_{1A}$ receptors is the principal mechanism of action of xaliproden and provide further support for the utility of 5-HT$_{1A}$ receptor activation as an anti-nociceptive strategy.
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Keywords: xaliproden; 5-HT$_{1A}$; nociception; pain management; rat; WAY100635

Abbreviations: AAALAC Intl., Association for the Assessment and Accreditation of Laboratory Animal Care International; ANOVA, analysis of variance; CHO, Chinese hamster ovary; DG, dentate gyrus; Ent(sup), superficial layers of entorhinal cortex; Ent(deep), deep layers of entorhinal cortex; Fr, frontal cortex; [^{35}S]GTPγS, guanosine 5′-O-(3-[^{35}S]thio)triphosphate; Hi, hippocampus; HPLC, high performance liquid chromatography; i.p., intraperitoneal; LS, lateral septum; p.o., per os; s.c., subcutaneous

Introduction

Xaliproden (SR57746A; 1-[2-(naphth-2-yl)ethyl]-4-(3-trifluoromethylphenyl))-1,2,5,6 tetrahydropyridine hydrochloride) is a selective and potent 5-HT$_{1A}$ receptor agonist with low nanomolar affinity at 5-HT$_{1A}$ receptors and an efficacy for activating these receptors equivalent to that of (±)8-OH-DPAT (Bachy *et al.*, 1993; Cervo *et al.*, 1994). In tests predictive of antidepressant properties, including the forced swim and learned helplessness tests, xaliproden reduced immobility scores and increased the duration of escape ('struggling') behaviour, especially when given in a semi-chronic regimen (Simiand *et al.*, 1993; Cervo *et al.*, 1994). Xaliproden is also

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active in a number of 'anxiolytic-like' activity tests including a Geller-Seifter conflict test, the staircase and the lithium chloride taste aversion models (Simiand *et al.*, 1993).

Later studies of xaliproden focused on pro-neurotrophic properties of this compound in cellular models including rat pheochromocytoma PC12 cells (Pradines *et al.*, 1995; Fournier *et al.*, 1998), primary neuronal cells (Ruigt *et al.*, 1996; Fournier *et al.*, 1998; Magazin *et al.*, 1998; Duong *et al.*, 1999), and in animal models of neurodegeneration (Duong *et al.*, 1998; Iwasaki *et al.*, 1998; Lemaire *et al.*, 2002). These studies suggested that these effects of xaliproden were not due to activation of 5-HT$_{1A}$ receptors because other 5-HT$_{1A}$ agonists [buspirone and (±)8-OH-DPAT] did not share these pro-neurotrophic properties either *in vitro* (Pradines *et al.*, 1995) or *in vivo* (Fournier *et al.*, 1993). However, more recent data suggest that agonism at 5-HT$_{1A}$ receptors may be responsible for these effects of xaliproden (Appert-Collin *et al.*, 2005a,b). Based on these pro-neurotrophic properties of xaliproden, clinical investigations have been conducted with this compound in different indications including amyotrophic lateral sclerosis (Meininger *et al.*, 2004), Alzheimer's disease (Price *et al.*, 2007) and, more recently, on chemotherapy-induced peripheral neuropathy in colorectal cancer patients (Susman, 2006; Wolf *et al.*, 2008).

The latter clinical trials are of particular interest in view of the recent demonstration in preclinical studies that high-efficacy 5-HT$_{1A}$ receptor agonists reduce acute tonic nociceptive pain (Bardin *et al.*, 2003), but are also active in models of neuropathic pain (Colpaert, 2006; Deseure *et al.*, 2007). Interestingly, in contrast to opiates, long-term management of pain with high-efficacy 5-HT$_{1A}$ receptor agonists shows inverse tolerance (Colpaert, 2006), which means that pain management improves with chronic treatments. 5-HT$_{1A}$ receptor agonists may thus be well adapted for the management of chronic pain.

5-HT$_{1A}$ receptors are expressed both as inhibitory autoreceptors on serotoninergic neurons and post-synaptically on different neuronal populations (Barnes and Sharp, 1999). Blockade of 5-HT$_{1A}$ autoreceptors has been associated with anti-nociception by general augmentation of 5-hydroxytryptaminergic tone (see Mico *et al.*, 2006), while direct activation of post-synaptic 5-HT$_{1A}$ heteroreceptors in other structures of the CNS may also reduce pain. In the spinal cord, 5-HT$_{1A}$ receptors are located on primary afferent fibres from dorsal root ganglia (Daval *et al.*, 1987) and on intrinsic neurons of the dorsal horn (Pompeiano *et al.*, 1992; Zhang *et al.*, 2002). Activation of spinal cord 5-HT$_{1A}$ receptors inhibits neuronal activity (Garraway and Hochman, 2001), and intrathecal injection of 5-HT$_{1A}$ receptor agonists is anti-nociceptive (Oyama *et al.*, 1996; Nadeson and Goodchild, 2002; Bardin and Colpaert, 2004). Stimulation of 5-HT$_{1A}$ receptors located at supra-spinal levels may also indirectly modulate pain. For example, stimulation of these receptors at the level of the brainstem activates descending anti-nociceptive noradrenergic pathways (Millan, 2002). 5-HT$_{1A}$ receptors are also present in thalamic nuclei where they can indirectly activate inhibitory outputs from the periaqueductal grey to the spinal cord (Xiao *et al.*, 2005). Finally, stimulation of cortical 5-HT$_{1A}$ receptors may also modulate pain perception (Kharkevich and Churukanov, 1999). Taken together, these considerations highlight both the therapeutic potential of 5-HT$_{1A}$ receptor agonists and the interest of xaliproden as a ligand at this site. However, to the best of our knowledge, no data are available describing the activity of xaliproden in models of functional autoradiography, neurotransmitter release or pain. In addition, we are not aware of reports on interaction studies with the selective 5-HT$_{1A}$ antagonist WAY100635 to confirm the involvement of these receptors in the mechanism of action of xaliproden. The present study therefore investigated the affinity and activity of xaliproden on a range of monoaminergic receptors, on rat brain autoradiography of G-protein activation as well as on *in vivo* 5-HT$_{1A}$ receptor occupancy.

Moreover, in microdialysis studies, 5-HT$_{1A}$ receptor agonists increase extracellular levels of dopamine in rat frontal cortex by stimulating post-synaptic 5-HT$_{1A}$ heteroreceptors, while reducing 5-HT levels in hippocampus via pre-synaptic 5-HT$_{1A}$ autoreceptors stimulation (Assié *et al.*, 2008). These two measures may allow identification of 5-HT$_{1A}$ agonists with either pre- or post-synaptic preference (Assié *et al.*, 2006; Newman-Tancredi *et al.*, 2008). The effects of xaliproden on extracellular cortical dopamine and hippocampal 5-HT levels were thus evaluated by microdialysis in freely moving rats.

We used injection of formalin into the hindpaw of adult rats as a model of acute tonic nociceptive pain, as we have characterized the anti-nociceptive properties of several high-efficacy 5-HT$_{1A}$ receptor agonists in this model (Bardin *et al.*, 2003). This model produces moderate pain generated by injured tissue that has two distinct phases: an early (0–10 min post formalin injection) and a late (15–60 min post formalin injection) phase of paw-licking/flinching and paw elevation (Tjolsen *et al.*, 1992; Bardin *et al.*, 2003; Sawynok and Liu, 2004). Whereas pain-related behaviours in the early phase reflect a direct activation of nociceptors, the late phase involves ongoing inflammation and central sensitization (Sawynok and Liu, 2004), these latter phenomena being also present in neuropathic pain (Zimmermann, 2001; Moalem and Tracey, 2006).

In most tests, the effects of xaliproden were also evaluated in interaction with WAY100635 to confirm the involvement of 5-HT$_{1A}$ receptors. Part of this work has been presented in an abstract form (Martel *et al.*, 2007).

Methods

Animals

All animals were purchased from Charles River Laboratories (L'Arbresle, France) and were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC Intl.)-accredited facility in strict compliance with all applicable regulations. In addition, the experimental protocols were carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research. Animals were housed in the animal-keeping facilities, under controlled conditions (12/12 h light/dark cycle: lights on at 7:00 AM; ambient temperature 21 ± 1°C; humidity 55 ± 5%), with rodent food and filtered (0.2 μm pore diameter) tap water available *ad libitum*. At least 4 days was allowed for adaptation before the animals were used in experiments.

Receptor binding assays

Competition binding experiments were carried out by using radioligands, buffers and incubation conditions described previously (Newman-Tancredi *et al.*, 2007). Experiments were carried out in duplicate and repeated at least three times. All binding experiments were terminated by rapid filtration, through GF-B fibre 96-well unifilters (Perkin Elmer Life Science, Courtaboeuf, France) followed by three rapid rinses with cold (4°C) buffer. Radioactivity retained on the filters was measured by liquid scintillation spectroscopy. Data were analysed by using sigmoid curve with variable slope from a non-linear curve-fitting programme (PRISM 4.03, GraphPad Software Inc., La Jolla, CA, USA). pK$_i$ values (= –Log[K_i]) were derived from competition binding isotherms by using the Cheng–Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/(1 + [Ligand]/K_D Ligand)$$

where IC$_{50}$ was the concentration of xaliproden, or (+)8-OH-DPAT necessary to displace 50% of the ligand specific binding, [Ligand] was the concentration of the radioligand, and K_DLigand was the affinity of the radioligand for the appropriate receptor.

Functional responses at native rat and recombinant human 5-HT$_{1A}$

The agonist properties of xaliproden at rat and human 5-HT$_{1A}$ receptors were determined *in vitro* by measurement of [^{35}S]GTPγS (guanosine 5'-O-(3-[^{35}S]thio)triphosphate) binding, a measure of G-proteins activation, on either membranes from rat hippocampus, or from HeLa or glioma C6 cells expressing recombinant human 5-HT$_{1A}$ receptors. Complete description of the methodologies, together with relevant literature references are described in Newman-Tancredi *et al*. (2007).

[^{35}S]GTPγS functional autoradiography

Frozen rat brains were cut horizontally in 20 µm thick serial sections and fixed on microscope slides. [^{35}S]GTPγS autoradiography was carried out in HEPES buffer as described in Newman-Tancredi *et al*. (2007). Quantifications were performed on sets of six serial sections including each of the following treatments: (i) 10 µmol·L^{-1} xaliproden + 10 µmol·L^{-1} WAY100635; (ii) 10 µmol·L^{-1} xaliproden; (iii) basal (no treatment); (iv) non-specific (10 µmol·L^{-1} GTPγS); (v) 10 µmol·L^{-1} (+)8-OH-DPAT; and (vi) 10 µmol·L^{-1} (+)8-OH-DPAT + 10 µmol·L^{-1} WAY100635. Brain structures known to contain high densities of 5-HT$_{1A}$ receptors (Pompeiano *et al.*, 1992) were quantified by film densitometry using commercial [^{14}C] standards (GE Healthcare). Values were normalized to the basal [^{35}S]GTPγS binding minus non-specific (defined as 100%) for each set and brain structure, and measurements were averaged for each animal. A total of six rat brains were used for these quantifications, and the mean ± SEM of the six animals are reported for each brain region and treatments. A one-way ANOVA (PRISM 4.03, GraphPad Software Inc., La Jolla, CA, USA) was used to test differences between treatments, followed by Tukey's multiple comparison *post hoc* tests, to evaluate reversal of the 5-HT$_{1A}$ agonist effects by WAY100635. In order to evaluate if the antagonist completely reversed the agonists effects, the differences from basal (100%) on sections co-treated with WAY100635 were also tested by a one sample Student's *t*-test.

In vivo *[^{3}H]WAY100635 binding*

Male NMRI mice, weighing 20–22 g upon arrival, were group-housed (12 mice per cage) with free access to food and water. On the day of the experiment, mice were housed individually. Vehicle (1% Tween 80 in water) or xaliproden (0.63, 2.5, 10 or 40 mg·kg^{-1}) were administered p.o. (per os) after which the animal was returned to its cage. Thirty minutes later, the mouse was gently introduced and maintained in a Plexiglas cylinder, with the tail protruding, and 48 pmol of a [^{3}H]WAY100635 solution (4 µCi in 0.2 mL) was slowly injected into the caudal vein and the mouse was again returned to its cage. One hour after the p.o. administration (or 30 min after [^{3}H]WAY100635 injection), the animal was decapitated, and frontal cortex and hippocampus were dissected out, weighed and frozen on dry ice. At the end of the experiment, the tissue was thawed and homogenized (10 s with an Ultra-Turrax) in 3 mL distilled water. Radioactivity in three 0.5 mL samples (triplicates) was counted with liquid scintillation spectroscopy using Emulsifier Safe scintillation liquid and a TriCarb 2500 scintillation counter (PerkinElmer Life Sciences, Courtaboeuf, France). Data were normalized to percentage of controls and are expressed as mean ± SEM. Data were analysed by using a sigmoid curve with variable slope equation from the PRISM 4.03 software to determine the ID$_{50}$. The maximum value of the curve was constrained at the value of control animals (100%). Dose–response data were analysed by a one-way ANOVA, followed by a Dunnett's *post hoc* test for comparison with the control (vehicle-administered) group.

Microdialysis

Male Sprague-Dawley rats, weighing 240–260 g upon arrival, were group-housed (three rats per cage) with free access to food and water. Under isoflurane anaesthesia, rats were implanted with a guide cannula positioned just above the frontal cortex or hippocampus as described before (Assié *et al.*, 2005). Following surgery, animals were housed individually with food and water freely available. At the end of the day, each rat was placed in a microdialysis cage. On the following morning, the dummy probe was replaced by a microdialysis probe (3 mm length, 0.5 mm diameter, CMA 12, CMA/Microdialysis, Solna, Sweden). The probe was continuously perfused (1.1 µL·min^{-1}) with artificial cerebrospinal fluid (containing 1 µmol·L^{-1} of the selective 5-HT reuptake inhibitor, citalopram for the measures of 5-HT). Starting approximately 2 h after probe implantation, samples were collected every 20 min. Analysis of dopamine or 5-HT was performed by means of an online high-performance liquid chromatography (HPLC) system with electrochemical detection. The perfusate levels of neurotransmitter are expressed as percentage of the mean of the absolute quantity of transmitter collected in the four pre-injection control samples (basal level). Animals were treated with either an i.p. injection of vehicle (1% Tween 80) or xaliproden (0.63, 2.5 or 10 mg·kg^{-1}), and microdialysis samples were collected each 20 min for another 140 min.

Data were analysed by using a repeated measures ANOVA carried out with the mixed procedure of SAS 8.2 software for PC (Littell *et al.*, 2000). Percentage measures taken after treatment administration were included in the statistical analysis of post-treatment effect (i.e. 20–140 min). *Post hoc* comparisons were made versus vehicle-treated animals with the method of contrasts based on the Fisher *post hoc* test.

Formalin pain test

Male Sprague-Dawley rats weighing 160–180 g upon arrival were group-housed (five animals per cage) with free access to food and water. The formalin test was carried out as described by Bardin *et al.* (2005). The recording of behaviour started immediately after subcutaneous (s.c.) injection of formalin (50 µL; 2.5% v/v in saline) into the plantar surface of the right hindpaw and lasted for 5 min ('early phase') while recording of the 'late phase' started at 22.5 min and also lasted for 5 min. Within these observation periods, rats were observed for the presence or absence of spontaneous pain behaviours every 30 s, that is: (i) the injected paw is elevated and not in contact with any surface; and (ii) the injected paw is licked. This observation cycle was repeated 10 times during the 5 min period. Thus, the incidence of a particular behaviour could vary from 0 to 10 for each of the two observation periods. Animals received either a single i.p. treatment with vehicle (0.9% saline) or xaliproden (0.63, 2.5 or 10 mg·kg^{-1}) 15 min before formalin, or a combination of antagonist plus agonist (s.c. injection of saline or 0.16 mg·kg^{-1} WAY100635 administered 45 min before the i.p. treatment with vehicle or 10 mg·kg^{-1} xaliproden). Data were analysed by a one- (vehicle vs. xaliproden alone) or two-way (WAY100635 interaction studies) ANOVA, followed by Dunnett's (vehicle vs. xaliproden alone) or Neuman-Keul's (WAY100635 interaction studies) *post hoc* tests for comparison with the appropriate controls.

Materials

Xaliproden (purity >99.5%, as assessed by HPLC) and WAY100635 (purity >99%, as assessed by HPLC) were synthesized by Jean-Louis Maurel from the Chemistry Department, Centre de Recherche Pierre Fabre. All other reagents were from Sigma-Aldrich (St. Quentin Fallavier, France) and were of the highest purity available. For *in vitro* assays, xaliproden was dissolved in 100% dimethyl sulphoxide while WAY100635 was dissolved in ultra-pure water, at a concentration of 10^{-3} mol·L^{-1}, and serial dilutions were performed in distilled water with a robot (Multiprobe, PerkinElmer Life Sciences, Courtaboeuf, France). For *in vivo* assays, drugs were either dissolved in saline or suspended in 1% Tween 80 in distilled water (two drops Tween 80 in 10 mL water) and administered p.o. (saline), injected s.c. (saline) or i.p. (1% Tween 80) in a volume of 10 mL·kg^{-1}. Doses refer to the weight of the free base.

Radioligands including [^{3}H]8-OH-DPAT (TRK.850: 160–240 Ci·mmol^{-1}), [^{3}H]GR125,743 (TRK.1046: 50–86 Ci·mmol^{-1}), [^{3}H]mesulergine (TRK.845: 70–85 Ci·mmol^{-1}), [^{3}H]SCH 23390 (TRK.876: 60–90 Ci·mmol^{-1}), [^{3}H]RX 821002 (TRK.914: 40–70 Ci·mmol^{-1}), [^{3}H]citalopram (TRK.1068: 60–86 Ci·mmol^{-1}), [^{3}H]prazosin (TRK.843: 65–85 Ci·mmol^{-1}), [^{3}H]spiperone (TRK.818: 78–93 Ci·mmol^{-1}), [^{35}S]GTPγS (1000–1200 Ci·mmol^{-1}) and WAY100635 (TRK.1034: 60–86 Ci·mmol^{-1}) were purchased from GE Healthcare (Aulnay Sous Bois, France) while [^{3}H]ketanserin (NET-791: 60–90 Ci·mmol^{-1}) and [^{3}H]YM-09151-2 (NET-1004: 70–87 Ci·mmol^{-1}) were purchased from PerkinElmer Life Sciences (Courtaboeuf, France).

Experiments with native rat receptors employed membranes prepared from frozen brains that were purchased from Charles River Laboratories (L'Arbresle, France; animals weighed 180–200 g at time of death) and stored at –70°C before use. Binding experiments at recombinant human receptors were carried out by using membranes from Chinese hamster ovary cell lines stably expressing monoamine receptors and purchased from PerkinElmer Life Sciences (Courtaboeuf, France), while h5-HT$_{1A}$-C6 glia and h5-HT$_{1A}$-HeLa cell lines were grown and maintained in house.

Results

In vitro *binding*

Xaliproden had high affinity at 5-HT$_{1A}$ receptors (pK$_i$ = 8.84 and 9.00 at rat and human receptors respectively; Table 1). It also had a high selectivity (>2000-fold) for 5-HT$_{1A}$ receptors when compared with the other 5-HT receptor subtypes tested, while showing moderate affinity at rat dopamine D$_2$ receptors (pK$_i$ = 6.78; 115-fold selectivity for r5-HT$_{1A}$) and α_1 adrenoceptors (pK$_i$ = 6.61; 170-fold selectivity). The affinity of xaliproden for 5-HT$_{1A}$ receptors was comparable to that of the prototypical selective 5-HT$_{1A}$ receptor agonist (+)8-OH-DPAT (pK$_i$ = 9.15 and 9.50 at rat and human 5-HT$_{1A}$ receptors respectively).

Table 1 Affinities of xaliproden and (+)8-OH-DPAT at rat (r) and human (h) receptor sites derived from binding assays

Receptor	*Xaliproden*	*(+)8-OH-DPAT*
r5-HT$_{1A}$ (Hi)	8.84 ± 0.20 (3)	9.15 ± 0.11 (3)
r5-HT$_{1A}$ (Cx)	8.16 ± 0.13 (3)	8.66 ± 0.07 (3)
r5-HT$_{1B}$ (Cx)	<5 (2)	<5 (2)
r5-HT$_{2A}$ (Cx)	<5 (2)	<5 (2)
rD$_1$ (Str)	<5 (2)	<5 (2)
rD$_2$ (Str)	6.78 ± 0.15 (3)	6.08 ± 0.03 (3)
rα_1 (Cx)	6.61 ± 0.08 (3)	5.82 ± 0.11 (3)
rα_2 (Cx)	<5 (3)	6.52 ± 0.04 (3)
h5-HT$_{1A}$ (CHO)	9.00 ± 0.03 (3)	9.50 ± 0.03 (3)
h5-HT$_{2A}$ (CHO)	5.62 ± 0.10 (3)	5.84 ± 0.02 (3)
h5-HT$_{2B}$ (CHO)	5.64 ± 0.08 (2)	5.46 ± 0.05 (3)
h5-HT$_{2C}$ (CHO)	<5 (2)	<5 (3)
hD$_1$ (CHO)	<5 (3)	<5 (3)
hD$_{2S}$ (CHO)	<5 (3)	<5 (4)
hD$_{2L}$ (CHO)	<5 (3)	n.d.
hD$_3$ (CHO)	<5 (3)	6.58 ± 0.11 (3)
hD$_{4.4}$ (CHO)	6.23 ± 0.04 (3)	<5 (3)
hM$_1$ (CHO)	<5 (2)	<5 (3)
hM$_3$ (CHO)	<5 (2)	<5 (3)
hH$_1$ (CHO)	5.72 ± 0.16 (3)	<5 (3)

Data are expressed as pK$_i$ ± SEM of (n) determinations.
α_1, α_2, adrenoceptors; CHO, membranes from Chinese hamster ovary cells transfected with the human receptor specified; Cx, cortical membranes; D, dopamine (D$_{2S}$, D$_{2L}$, short or long form of D$_2$ receptor); H, histamine; Hi, hippocampal membranes; M, muscarinic; n.d, not determined; Str, striatal membranes (nomenclature follows Alexander *et al.*, 2008).

Table 2 Potencies (pEC_{50}) and efficacies (E_{MAX}) of xaliproden and (+)8-OH-DPAT at rat (r) and human (h) 5-HT$_{1A}$ receptors ([^{35}S]GTPγS assays)

Membrane preparation	*Xaliproden*		*(+)8-OH-DPAT*	
	pEC_{50}	E_{MAX}	pEC_{50}	E_{MAX}
r5-HT$_{1A}$ (Hi)	7.58 ± 0.02	61 ± 1 (3)	6.93 ± 0.03	67 ± 5 (3)
h5-HT$_{1A}$ (C6)	7.39 ± 0.05	62 ± 2 (3)	7.16 ± 0.09	55 ± 2 (3)
h5-HT$_{1A}$ (HeLa)	7.24 ± 0.10	93 ± 4 (3)	7.17 ± 0.05	107 ± 3 (3)

Data are expressed as the mean ± SEM of (*n*) determinations. E_{MAX} are expressed as percentage of the response above basal observed with 10 μmol·L^{-1} of 5-HT. [^{35}S]GTPγS, guanosine 5′-O-(3-[^{35}S]thio)triphosphate; C6, membranes from rat glioma C6 cell line transfected with human 5-HT$_{1A}$ receptors; HeLa, membranes from HeLa cell line (human) transfected with human 5-HT$_{1A}$ receptors; Hi, hippocampal membranes.

In vitro *functional assays*

The agonist properties of xaliproden at 5-HT$_{1A}$ receptor were comparable to those of (+)8-OH-DPAT (Table 2). In all three assays, xaliproden had potencies [pEC_{50} = 7.58, 7.39 and 7.24 at rat hippocampal 5-HT$_{1A}$ receptors, and human 5-HT$_{1A}$ receptors (expressed in either glioma C6 or HeLa cells respectively)] slightly superior to those of (+)8-OH-DPAT (pEC_{50} = 6.93, 7.16 and 7.17 respectively). Efficacies of xaliproden (E_{MAX} = 61%, 62% and 93% respectively) were comparable to those of (+)8-OH-DPAT (67%, 55% and 107% respectively). These assays demonstrate that xaliproden is a potent and efficacious 5-HT$_{1A}$ receptor agonist.

[^{35}S]GTPγS functional autoradiography

Xaliproden (10 μmol·L^{-1}) elicited a net increase in [^{35}S]GTPγS radioactivity bound in structures known to contain high densities of 5-HT$_{1A}$ receptor subtypes (Figure 1, upper right panel), including lateral septum, hippocampus, dentate gyrus, frontal cortex and superficial and deep layers of entorhinal cortex. These effects of xaliproden were completely reversed by co-application of 10 μmol·L^{-1} WAY100635 (Figure 1, lower right panel). Quantifications of [^{35}S]GTPγS radioactivity bound in these structures are summarized in Figure 2. ANOVA revealed highly significant changes in [^{35}S]GTPγS radioactivity bound in all the above-mentioned structures when comparing treatments [lateral septum: $F(3,23) = 48.1$, $P < 0.0001$; hippocampus: $F(3,23) = 54.8$, $P < 0.0001$; dentate gyrus: $F(3,23) = 51.6$, $P < 0.0001$; frontal cortex: $F(3,23) = 9.9$, $P < 0.001$; superficial: layers of entorhinal cortex: $F(3,23) = 29.1$, $P < 0.0001$ and deep layers of entorhinal cortex: $F(3,23) = 32.1$, $P < 0.0001$]. While co-treatment with 10 μmol·L^{-1} WAY100635 completely reversed the effect of 10 μmol·L^{-1} xaliproden in all structures, it only partially reversed that of 10 μmol·L^{-1} (+)8-OH-DPAT (Figure 2).

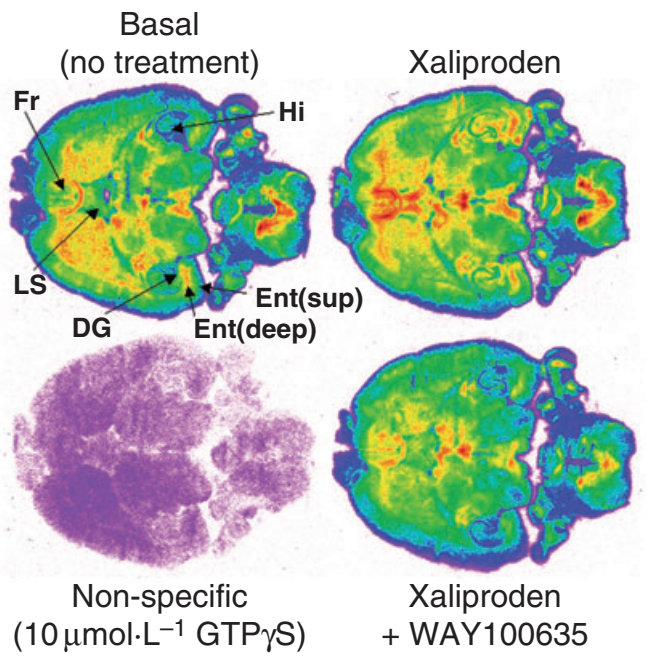

Figure 1 Influence of xaliproden (10 μmol·L^{-1}) with or without WAY100635 (10 μmol·L^{-1}) on G-protein activation in horizontal brain sections of rats as assessed by [^{35}S]GTPγS (guanosine 5′-O-(3-[^{35}S]thio)triphosphate) functional autoradiography. Pseudo-colour images of sections incubated with [^{35}S]GTPγS and drug treatments: blue/pink indicates low levels of activation while red represents high activation. Images are from a representative experiment. DG, dentate gyrus; Hi, hippocampus; LS, lateral septum; frontal (Fr) and entorhinal (Ent) cortices, the latter structure being divided into superficial (sup) and deep layers.

Inhibition of in vivo *[^{3}H]WAY100635 binding in mouse frontal cortex and hippocampus*

5-HT$_{1A}$ receptors in mouse brain were labelled *in vivo* with [^{3}H]WAY100635 (Figure 3). Control (saline-treated) animals exhibited radio-labelling of 476 ± 17 and 422 ± 15 dpm·mg^{-1} wet weight in frontal cortex and hippocampus respectively. Xaliproden (0.63–40 mg·kg^{-1}, p.o.) significantly and dose-dependently inhibited the *in vivo* binding of [^{3}H]WAY100635 in frontal cortex [$F(4,27) = 27.0$; $P < 0.0001$] and hippocampus [$F(4,27) = 28.7$; $P < 0.0001$] of mice with ID_{50} of 3.5 (95% CI: 0.6–20.4) and 3.3 (95% CI: 1.8–5.8) mg·kg^{-1}, p.o. respectively. The maximal inhibition of labelling by xaliproden was 64 ± 14% and 53 ± 8% in frontal cortex and hippocampus, respectively, these maximal values being comparable to those observed with (+)-8-OH-DPAT (160 mg·kg^{-1}, p.o.; data not shown).

Microdialysis measurements of extracellular dopamine in frontal cortex and 5-HT in hippocampus

The mean basal extracellular concentration of dopamine in the rat medial prefrontal cortex was 8.19 ± 0.50 fmol in 20 μL (n = 30). Xaliproden (0.63–10 mg·kg^{-1}, i.p.) dose-dependently increased dopamine levels (Figure 4 top panels). There was a significant effect of treatment [$F(5,24) = 4.7$, $P < 0.01$] and a

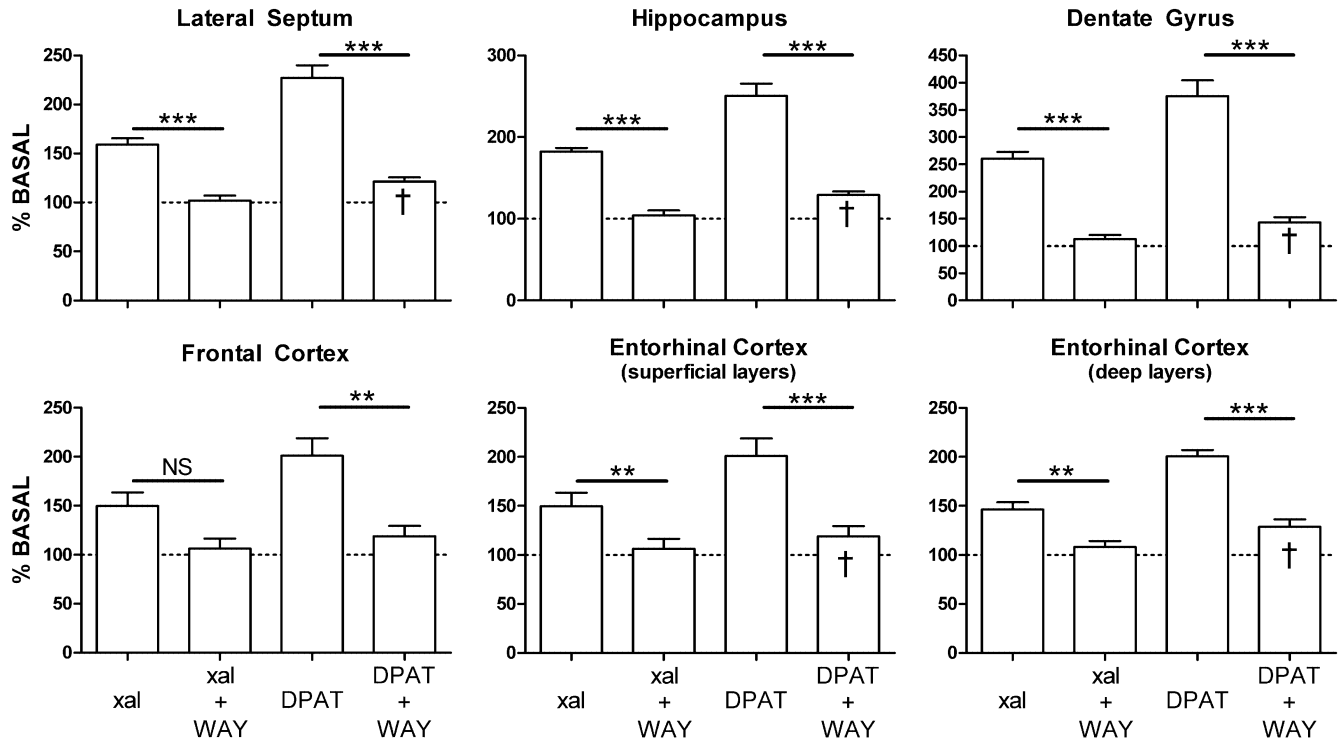

Figure 2 Quantification of the effects of 10 μmol·L^{-1} xaliproden (xal) or 10 μmol·L^{-1} (+)8-OH-DPAT (DPAT), and their reversal by co-treatment with 10 μmol·L^{-1} WAY100635 (WAY), on [^{35}S]GTPγS labelling of rat brain structures. Bars are means ± SEM of average measurements for each animal/brain structure performed on serial brain sections from six animals. Statistics: $^{**}P < 0.01$, $^{***}P < 0.001$, Tukey's *post hoc* test following significant one-way ANOVA; †$P < 0.01$, one sample *t*-test versus basal (100%; dotted line). NS, not significant.

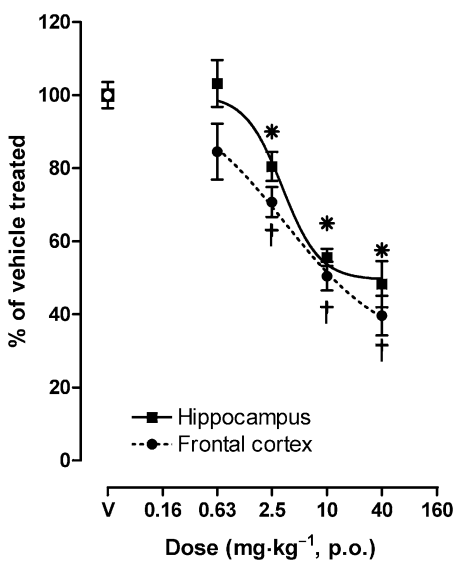

Figure 3 Effects of xaliproden on *in vivo* [^{3}H]WAY100635 binding in the mouse brain. Values are expressed as the percentage of the average for the vehicle-treated group. Symbols are mean ± SEM of five animals per treatment group. *$P < 0.05$ (hippocampus); †$P < 0.05$ (frontal cortex), versus vehicle-treated group, Dunnett's *post hoc* test following significant one-way ANOVA. V, vehicle (1% Tween 80 in distilled water).

significant 'treatment × time' interaction [$F(30,142) = 1.9$, $P < 0.01$], but no significant effect of time alone was detected [$F(6,142) = 1.2$, $P > 0.05$]. Compared with controls, xaliproden produced a significant increase in overall extracellular dopamine at 2.5 and 10 mg·kg^{-1} ($P < 0.01$) with an estimated ED_{50} of 0.7 mg·kg^{-1}. The selective 5-HT$_{1A}$ receptor antagonist, WAY100635 (0.16 mg·kg^{-1}, s.c.) administered 40 min before xaliproden (2.5 mg·kg^{-1}, i.p.) significantly attenuated the effects of the latter ($P < 0.01$). The mean basal extracellular concentration of 5-HT in the rat ventral hippocampus was 48.5 ± 1.8 fmol in 20 μL ($n = 30$) in the presence of 1 μmol·L^{-1} of the 5-HT reuptake inhibitor, citalopram. Xaliproden (0.63–10 mg·kg^{-1}, i.p.) dose-dependently decreased 5-HT levels (Figure 4 bottom panels). There was a significant effect of time [$F(6,139) = 6.0$, $P < 0.0001$] and treatment [$F(5,24) = 4.1$, $P < 0.01$] and a significant 'treatment × time' interaction [$F(30,139) = 2.0$, $P < 0.01$]. Compared with controls, xaliproden produced a significant decrease in overall extracellular 5-HT at 2.5 and 10 mg·kg^{-1} ($P < 0.05$) with an estimated ED_{50} of 1.2 mg·kg^{-1}. WAY100635 (0.16 mg·kg^{-1}, s.c.) administered 40 min before xaliproden (10 mg·kg^{-1}, i.p.) also significantly attenuated the response to xaliproden ($P < 0.01$).

Anti-nociceptive effects in the formalin test

An i.p. injection of xaliproden (0.63–10 mg·kg^{-1}) induced dose-dependent and significant inhibitory effects on both paw elevation and licking, these effects being observed during both the early and late phases [paw elevation: $F(3,31) = 79.5$; $P < 0.001$ and $F(3,31) = 22.7$; $P < 0.001$ on early and late phase respectively; paw licking: $F(3,31) = 39.4$; $P < 0.001$ and $F(3,31) = 107.6$; $P < 0.001$ respectively; Figure 5]. The score of paw licking in the two phases was significantly reduced at the doses of 0.63 and 2.5 mg·kg^{-1}, whereas xaliproden totally

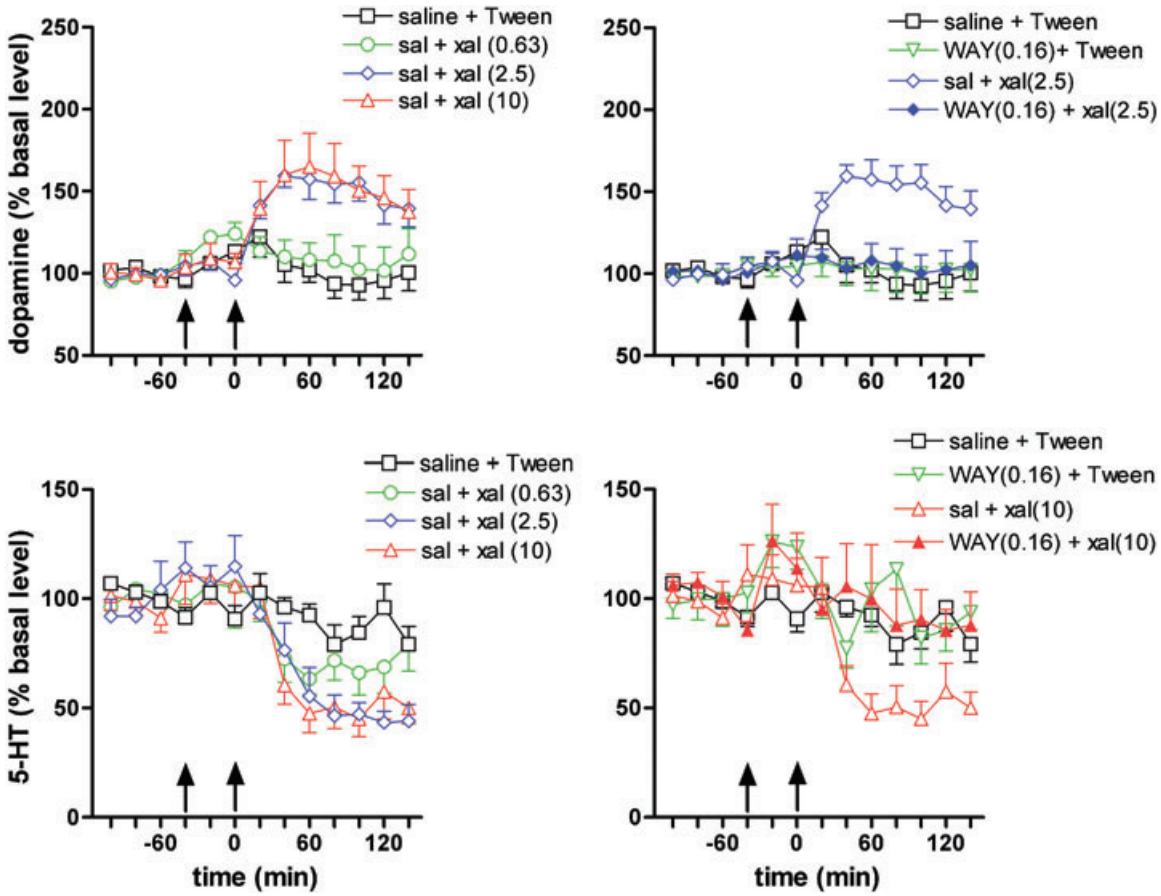

Figure 4 Effects of xaliproden alone (left panels) and in combination with the 5-HT$_{1A}$ antagonist WAY100635 (right panels) on extracellular levels of dopamine in the prefrontal cortex (top panels) and of 5-HT in the hippocampus (bottom panels) in rats. On each graph, the first arrow indicates s.c. injection of saline (sal) or WAY100635 (WAY), the second arrow indicates i.p. injection of vehicle (Tween) or xaliproden (xal). Doses (in mg·kg^{-1}) are given in parentheses. Values are expressed as percentage of the mean absolute amount of transmitter collected in the four samples before treatments (basal level = 100%). Symbols are mean ± SEM of five animals per treatment group.

inhibited paw licking at 10 mg·kg^{-1}. The score of paw elevation was also significantly reduced by xaliproden in the two phases, from 2.5 mg·kg^{-1} onward; total inhibition of paw elevation was obtained at 10 mg·kg^{-1} (Figure 5).

Pretreatment with WAY100635 (0.63 mg·kg^{-1}, s.c.) 45 min before i.p. injection of 10 mg·kg^{-1} of xaliproden completely blocked the anti-nociceptive effects of this dose of xaliproden (Figure 6). Two-way ANOVA found that in each phase, the pretreatment {paw elevation: early [$F(1,24) = 85.1$; $P < 0.001$] and late [$F(1,24) = 1005$; $P < 0.001$] phases, and paw licking: early [$F(1,24) = 70.9$; $P < 0.001$] and late [$F(1,24) = 78.2$; $P < 0.001$] phases} and the treatment {paw elevation: early [$F(1,24) = 85.1$; $P < 0.001$] and late [$F(1,24) = 1005$; $P < 0.001$] phases, and paw licking: early [$F(1,24) = 37.3$; $P < 0.001$] and late [$F(1,24) = 78.2$; $P < 0.001$] phases} interacted significantly {paw elevation: early [$F(1,24) = 91.7$; $P < 0.001$] and late [$F(1,24) = 1005$; $P < 0.001$] phases, and paw licking: early [$F(1,24) = 60.2$; $P < 0.001$] and late [$F(1,24) = 49.2$; $P < 0.001$] phases}. Subsequent multiple comparisons analysis showed that xaliproden significantly inhibited paw elevation and paw licking after vehicle pretreatment, while pretreatment with 0.63 mg·kg^{-1} WAY100635 completely antagonized the reduction of the paw elevation and paw licking score induced by 10 mg·kg^{-1} xaliproden (i.e. scores were not statistically different from saline-treated animals; see Figure 6). In subsequent experiments a lower dose of WAY100635 (0.16 mg·kg^{-1}) was tested and produced a similar reversal of the anti-nociceptive effects of 10 mg·kg^{-1} xaliproden [average scores ± SEM: 10 ± 0 (paw elevation, early phase), 9.9 ± 0.2 (paw elevation, late phase), 5.6 ± 0.9 (paw licking, early phase) and 8.3 ± 0.5 (paw licking, late phase)] to that obtained with 0.63 mg·kg^{-1} WAY100635 (see Figure 6). Indeed, effects of either doses of WAY100635 were not statistically different (unpaired Student's *t*-test: all $|t| < 1.6$; all $P > 0.05$). In addition, scores obtained within the 0.16 mg·kg^{-1} WAY100635 + saline treatment group [average scores ± SEM: 9.6 ± 0.4 (paw elevation, early phase), 10 ± 0 (paw elevation, late phase), 6.6 ± 0.9 (paw licking, early phase) and 7.5 ± 0.7 (paw licking, late phase)] were not statistically different from the saline + saline treatment group (unpaired Student's *t*-test: all $|t| < 1.4$; all $P > 0.05$).

Discussion

Despite the fact that xaliproden has been substantially characterized in clinical trials, preclinical information is lacking regarding its activity in models of G-protein activation,

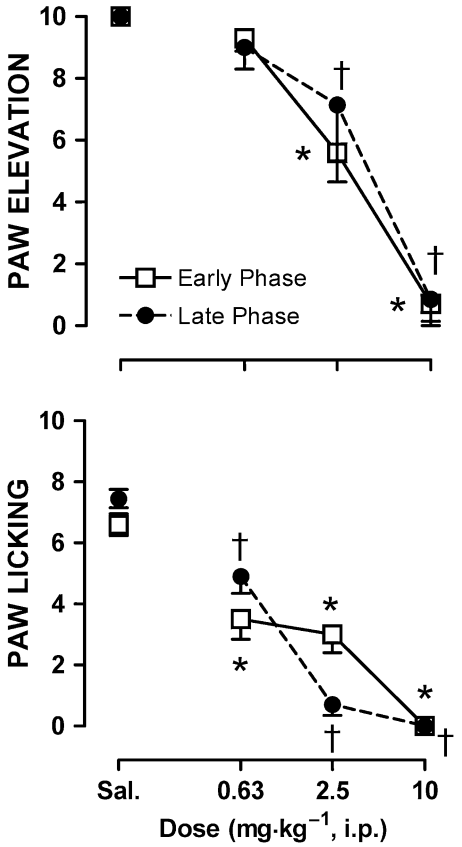

Figure 5 Effects of xaliproden on the paw elevation and paw licking behaviour during the two phases of the formalin test in rats. Symbols represent the means ± SEM score of seven animals per treatment group. $P < 0.05$ as compared with saline controls in early (*) and late (†) phases, Dunnett's *post hoc* test following significant one-way ANOVA. Sal., saline.

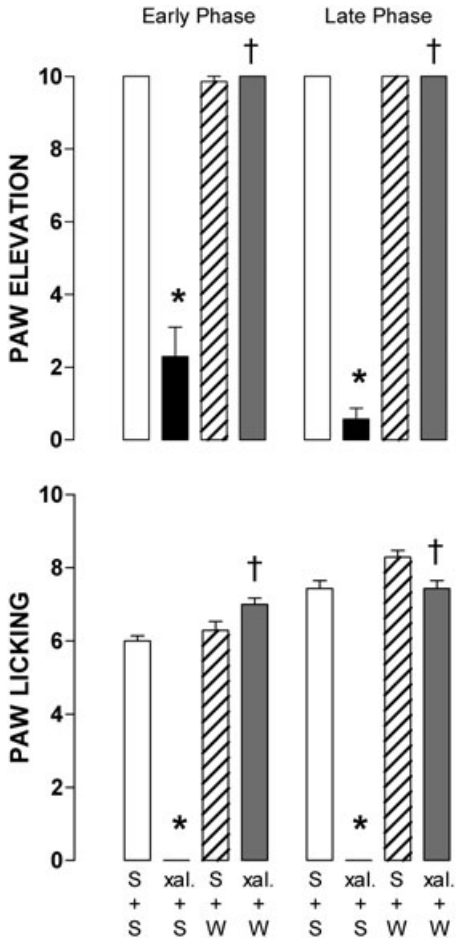

Figure 6 Effects of pretreatment with WAY100635 (W; 0.63 mg·kg^{-1}; s.c.) on the anti-nociceptive activity of xaliproden (xal.; 10 mg·kg^{-1}; i.p.) on the paw elevation and paw licking behaviours in the early (left panels) or late (right panels) phases of the formalin test in rats. Bars represent the mean ± SEM scores of seven animals per treatment group. *$P < 0.05$ versus saline + saline group, †$P < 0.05$ versus saline + xaliproden group, Neuman-Keul's *post hoc* test following significant ANOVA (see results for details). S, saline.

microdialysis and nociception. The present study therefore characterized its activity in a series of *in vitro* and *in vivo* pharmacological models. The principal findings are: (i) xaliproden is a relatively selective and potent 5-HT$_{1A}$ receptor agonist; (ii) xaliproden decreased hippocampal extracellular levels of 5-HT (controlled by pre-synaptic 5-HT$_{1A}$ receptors) as well as increased cortical dopamine levels (controlled by post-synaptic 5-HT$_{1A}$ receptors), effects that were blocked by the selective 5-HT$_{1A}$ receptor antagonist WAY100635; and (iii) xaliproden exerted anti-nociceptive influence in a model of acute tonic nociceptive and inflammatory pain, an effect that is also reliant on stimulation of 5-HT$_{1A}$ receptors.

Pharmacological profile of xaliproden

In a 'receptogram' of binding affinities, xaliproden showed marked selectivity for 5-HT$_{1A}$ receptors: affinity at other receptors was at least 100-fold lower than that measured at 5-HT$_{1A}$ receptors, with dopamine D$_2$-like receptors and α_1-adrenoceptors representing the principal 'secondary' targets. In [^{35}S]GTPγS binding-based functional assays for measuring activity at 5-HT$_{1A}$ receptors, xaliproden was as potent and efficacious as (+)8-OH-DPAT. Previous *in vitro* characterization of xaliproden using inhibition of forskolin-stimulated cAMP production (Bachy *et al.*, 1993; Cervo *et al.*, 1994) suggested that this drug had lower potency but equal efficacy as that of (±)8-OH-DPAT (Cervo *et al.*, 1994). The present data confirm that xaliproden is an efficacious 5-HT$_{1A}$ receptor agonist and indicated a slightly higher potency than (+)8-OH-DPAT in rat tissue. The selectivity of xaliproden for stimulating 5-HT$_{1A}$ receptors was also demonstrated by the pattern of activation of [^{35}S]GTPγS labelling on rat brain sections. The latter is consistent with activation of 5-HT$_{1A}$ receptors, because the labelled brain structures included lateral septum, hippocampus, frontal and entorhinal cortices, which are known to contain substantial densities of these sites (Pompeiano *et al.*, 1992). This was further confirmed by the fact that this pattern of [^{35}S]GTPγS labelling elicited by xaliproden was completely prevented by co-application of WAY100635. The partial reversal of (+)-8-OH-DPAT effects by 10 µmol·L^{-1} WAY100635 may be due to the use of a sub-maximal concentrations of WAY100635 for blocking 5-HT$_{1A}$ receptors under these conditions, or to actions of (+)-8-OH-DPAT at other receptor sites such as α_2-adrenoceptors for which (+)-8-OH-

DPAT shows modest agonist properties (Bonaventure *et al*., 2004). Finally, the direct interaction of xaliproden with 5-HT$_{1A}$ receptors was demonstrated *in vivo*, where it inhibited [^{3}H]WAY100635 binding to mouse frontal cortex and hippocampus. Taken together, these data provide compelling evidence for the selectivity of xaliproden for 5-HT$_{1A}$ receptors and its agonist properties at these sites.

The effects of xaliproden on fronto-cortical extracellular dopamine levels, as measured by microdialysis in freely moving animals, are also consistent with 5-HT$_{1A}$ receptor agonist properties. Xaliproden also reduced extracellular 5-HT levels in hippocampus with an ED$_{50}$ similar to that for elevating fronto-cortical dopamine, thus suggesting that xaliproden, in contrast to F15599, a highly selective prototypical post-synaptic 5-HT$_{1A}$-prefering compound (Assié *et al*., 2006; Newman-Tancredi *et al*., 2009), has no clear preference for either pre- or post-synaptic 5-HT$_{1A}$ receptors. These effects of xaliproden on extracellular neurotransmitter levels were antagonized by pretreatment with WAY100635, confirming that these two measures were mediated by 5-HT$_{1A}$ receptor activation. The ED$_{50}$ of these *in vivo* effects of xaliproden are consistent with the dosage at which antidepressant properties are observed [0.5 mg·kg^{-1} per day, p.o., b.i.d. × 5 days for reversal of learned helplessness (Simiand *et al*., 1993), and 0.3–3 mg·kg^{-1}, p.o. t.i.d. × 2 days for increasing 'struggling' and reducing 'floating' in the forced swim test (Cervo *et al*., 1994)].

Anti-nociceptive properties of xaliproden

Selective 5-HT$_{1A}$ receptor agonists have demonstrated anti-nociceptive properties in animal models of acute and chronic pain (Bardin *et al*., 2001; 2003; Nadeson and Goodchild, 2002; Colpaert, 2006), but no information is available concerning the activity of xaliproden in such models. We used s.c. injection of formalin into the plantar surface of the hindpaw in rats, as this test of acute tonic nociceptive pain provides a measure of both acute pain elicited immediately after formalin injection but also of a secondary phase associated with inflammation and neuroadaptative changes occurring at the level of the dorsal horn of the spinal cord (Sawynok and Liu, 2004). We have previously shown that this model of acute tonic nociceptive pain is well adapted for measuring the anti-nociceptive properties of 5-HT$_{1A}$ receptor agonists (Bardin *et al*., 2003). In this model, xaliproden reversed both paw licking and paw elevation in the same dose range as that at which it is active in other neurochemical and behavioural tests associated with 5-HT$_{1A}$ receptor stimulation (see above). Moreover, the anti-nociceptive effects of xaliproden were blocked by WAY100635, confirming that they were mediated by 5-HT$_{1A}$ receptor activation.

Few manifestations of the 'serotonin syndrome' (flat body posture and forepaw treading) were present following i.p. injection of up to 10 mg·kg^{-1} of xaliproden. The lack of significant serotonin syndrome at these doses is in agreement with reports that tremor and forepaw treading were only observed at very high doses of xaliproden (Simiand *et al*., 1993). Moreover, semi-chronic administration of xaliproden up to 9 mg·kg^{-1} per day over 3 days did not affect open-field locomotor activity (Cervo *et al*., 1994). We have previously shown that, induction of the serotonin syndrome could be dissociated from the anti-nociceptive properties of (+)8-OH-DPAT either by antagonism of α_1 adrenoceptors or by semi-chronic treatments (Bardin *et al*., 2001). These observations suggest that the anti-nociceptive effects of xaliproden, and more generally of high-efficacy 5-HT$_{1A}$ receptor agonists, are dissociated from non-specific behavioural interference, at least in the formalin test.

Xaliproden has given encouraging results in an initial phase III clinical trial for the treatment of chemotherapy-induced peripheral neuropathy in advanced colorectal cancer (Susman, 2006) and is currently being tested in a larger scale phase III study (Wolf *et al*., 2008). Xaliproden reduced intensity of peripheral neuropathy, without altering the effectiveness of the chemotherapy. The present study in a preclinical model of acute tonic nociceptive pain shows that, as other high-efficacy 5-HT$_{1A}$ receptor agonists, this compound also possesses anti-nociceptive properties. Chemotherapy-induced peripheral neuropathies comprise, among other things, increased pain sensation (Windebank and Grisold, 2008), and the present data suggest that part of the benefits of xaliproden against these neuropathies may be related to the anti-nociceptive properties of this drug. It would be interesting to examine this compound in preclinical models more closely related to neuropathic pain.

In conclusion, the present study shows that xaliproden exhibits relatively selective and marked agonist properties at 5-HT$_{1A}$ receptors. In all models where it was tested in interaction with the selective 5-HT$_{1A}$ receptor antagonist, WAY100635, activity of xaliproden was abolished, confirming mediation by 5-HT$_{1A}$ receptors. This was true of *in vitro* tests of G-protein activation on brain sections, *in vivo* neurotransmitter release experiments and anti-nociceptive activity in the formalin test. While 5-HT$_{1A}$ receptor agonists show potent anti-nociceptive properties that may be particularly well adapted to the management of chronic pain, preclinical evidence for neurotrophic, neuroprotective and antidepressant/anxiolytic activities suggest that these compounds may also have other therapeutic applications.

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Conflicts of interest

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