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RESEARCH PAPER

The activation of supraspinal GPR40/FFA1 receptor signalling regulates the descending pain control system

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BACKGROUND AND PURPOSE

The ω-3 polyunsaturated fatty acids exert antinociceptive effects in inflammatory and neuropathic pain; however, the underlying mechanisms remain unclear. Docosahexaenoic acid-induced antinociception may be mediated by the orphan GPR40, now identified as the free fatty acid receptor 1 (FFA1 receptor). Here, we examined the involvement of supraspinal FFA1 receptor signalling in the regulation of inhibitory pain control systems consisting of serotonergic and noradrenergic neurons.

EXPERIMENTAL APPROACH

Formalin-induced pain behaviours were measured in mice. Antinociception induced by FFA1 receptor agonists was examined by intrathecal injections of a catecholaminergic toxin, 5-HT lowering drug or these antagonists. The expression of FFA1 receptor protein and c-Fos was estimated by immunohistochemistry, and the levels of noradrenaline and 5-HT in the spinal cord were measured by LC-MS/MS.

KEY RESULTS

FFA1 receptors colocalized with NeuN (a neuron marker) in the medulla oblongata and with tryptophan hydroxylase (TPH; a serotonergic neuron marker) and dopamine β-hydroxylase (DBH; a noradrenergic neuron marker). A single i.c.v. injection of GW9508, a FFA1 receptor agonist, increased the number of c-Fos-positive cells and the number of neurons double-labelled for c-Fos and TPH and/or DBH. It decreased formalin-induced pain behaviour. This effect was inhibited by pretreatment with 6-hydroxydopamine, DL-p-chlorophenylalanine, yohimbine or WAY100635. Furthermore, GW9508 facilitated the release of noradrenaline and 5-HT in the spinal cord. In addition, GW1100, a FFA1 receptor antagonist, significantly increased formalin-induced pain-related behaviour.

CONCLUSION AND IMPLICATIONS

Activation of the FFA1 receptor signalling pathway may play an important role in the regulation of the descending pain control system.

Abbreviations

6-OHDA, 6-hydroxydopamine; DBH, dopamine β-hydroxylase; DHA, docosahexaenoic acid; GFAP, glial fibrillary acidic protein; i.t., intrathecal; LC, locus coeruleus; PCPA, DL-p-chlorophenylalanine; PUFA, polyunsaturated fatty acids; RVM, rostral ventromedial medulla; TPH, tryptophan hydroxylase

Tables of Links

TARGETS
GPCRs[a]
5-HT_{1A} receptors
α_2-Adrenoceptors
FFA1 receptors
Enzymes[b]
DBH, dopamine β-hydroxylase
TPH, tryptophan hydroxylase

LIGANDS
5-HT
DHA, docosahexaenoic acid
GW1100
GW9508
Noradrenaline
PCPA, p-chlorophenylalanine (fenclonine)
WAY100635
Yohimbine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013).

Introduction

Accumulating evidence has indicated that the ω-3 polyunsaturated fatty acids (PUFAs) are useful for the treatment of patients with pain (Tokuyama and Nakamoto, 2011). In fact, there is substantial evidence that PUFAs and their metabolites can suppress inflammatory pain, including pain associated with rheumatoid arthritis (Lee *et al.*, 2012) and joint pain (Goldberg and Katz, 2007) and headaches (Ramsden *et al.*, 2013). Hence, it is thought that the analgesic effects of PUFAs may result from anti-inflammatory activities mediated by the production of lipid mediators, such as D- or E-series resolvins (Ji *et al.*, 2011), the blockade of the arachidonic acid cascade (Gaudette and Holub, 1990), the suppression of inflammatory cytokines (Bagga *et al.*, 2003) and/or the inhibition of voltage-dependent sodium channels or calcium channels (Xiao *et al.*, 1995; Vreugdenhil *et al.*, 1996).

The orphan receptor GPR 40 has been identified as the free fatty acid receptor 1 (FFA1 receptor) and is activated by long-chain fatty acids, such as docosahexaenoic acid (DHA), arachidonic acid and eicosapentaenoic acid and is expressed predominantly in the CNS and in β-cells in the pancreatic islets (Briscoe *et al.*, 2003; Itoh *et al.*, 2003). Since 2003, most studies of FFA1 receptors concerned the insulin secretion mechanisms of the pancreas. However, more recently, expression of FFA1 receptors has been demonstrated in the brains of rodents (Nakamoto *et al.*, 2012a; Zamarbide *et al.*, 2014), and many studies of the functional role of these receptors in the brain have been carried out. For example, there have been several reports that FFA1 receptor signalling in the CNS may contribute to antidepressant effects (Nishinaka *et al.*, 2014) and to the generation of new neurons for learning and memory (Ma *et al.*, 2007; Ma *et al.*, 2008; Boneva and Yamashima, 2011). Our previous study has demonstrated that the i.c.v. administration of DHA or GW9508, which is a selective FFA1 receptor agonist, suppresses formalin-induced pain behaviour. It also attenuates complete Freund's adjuvant-induced mechanical allodynia and thermal hyperalgesia, suggesting that these effects occur by increasing β-endorphin release from pro-opiomelanocortin neurons (Nakamoto and Tokuyama, 2012b; Nakamoto *et al.*, 2012a; 2013). These results suggest that hypothalamic FFA1 receptors that are activated by free long-chain fatty acids might have an important role in pain control systems (Nakamoto *et al.*, 2013).

Generally, the sensation of pain is known to be modified by endogenous pain inhibitory systems, which act predominantly through descending noradrenaline and 5-HT systems and endogenous opioids, such as β-endorphin (Yoshimura and Furue, 2006). Interestingly, Mathieu *et al.* have reported that DHA enhances noradrenaline release by SH-SY5Y cells (Mathieu *et al.*, 2010). In addition, it has been reported that serotonergic neurotransmission increases in response to ω-3 PUFA administration in rats and that the density of 5-HT_{1A} receptors increases in response to ω-3 PUFAs. Furthermore, dietary deficiency of α-linolenic acid inhibits the activity of monoaminergic systems, including the serotonergic and noradrenergic systems (Kodas *et al.*, 2004; Chalon, 2006). Vines *et al.* have shown that ω-3 PUFA supplementation produces antidepressant effects that are mediated by an increase in 5-HT (Vines *et al.*, 2012). Thus, it is likely that brain FFA1 receptor signalling could be related to the monoaminergic pain control system.

In the present study, we examined the involvement of supraspinal FFA1 receptor signalling in the regulation of descending pain control systems that consist of serotonergic and noradrenergic neurons, in mice.

Experimental procedures

Animals

All animal care and experimental procedures were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals adopted by the Japanese Pharmacological Society and were approved by the Ethical Committee for Animal Experimentation of Kobe Gakuin University (approval number A13-23; Kobe, Japan). All studies involving animals are reported in accordance with the ARRIVE guide-

lines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 215 animals were used in the experiments described here.

Male ddY mice (4 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The ddY strain is an outbred one, and has been maintained as a closed colony. Mice were housed in cages at 23–24°C with a 12 h light–dark cycle (lights on from 8:00 am to 8:00 pm) and food and water available *ad libitum*.

Drug administration

A selective FFA1 receptor agonist, GW9508 (1.0 μg; Cayman Chemical Company, Ann Arbor, MI, USA), and a selective FFA1 receptor antagonist, GW1100 (10 μg; Cayman Chemical Company), were dissolved in 100% DMSO; Sigma-Aldrich Japan, Ishikari, Japan), and the solution was diluted with saline before the formalin test (0.2% DMSO final concentration). GW9508 and GW1100 were administered i.c.v., 10 min before the formalin test. The concentrations of GW9508 and GW1100 were chosen based upon our previous publication and others (Nakamoto *et al.*, 2012a). The mice were anaesthetized briefly with diethyl ether, and a needle (tip: 26-gauge, Natsume Seisakusyo Co., Ltd., Tokyo, Japan) attached to a 50 μL Hamilton microsyringe was inserted into a unilateral injection site to make a hole in the skull for injections. The drugs were administered in a volume of 5 μL through a disposable 27-gauge needle, which was inserted into the lateral ventricle (Haley and McCormick, 1957). The needle was inserted perpendicular to the skull. Each solution was injected without use of cannulae. While anaesthetized, some groups of mice were injected intrathecally (i.t.; Hylden and Wilcox, 1980) with 20 μg of the catecholaminergic neurotoxin 6-hydroxydopamine hydrobromide (6-OHDA; Sigma-Aldrich Japan K.K., Tokyo, Japan), 4 μg of yohimbine (Nacalai Tesque, Inc., Kyoto, Japan) or 10 μg of WAY100635 (Sigma-Aldrich Japan K.K.), which was dissolved in 5 μL of 0.9% saline containing ascorbic acid. The doses of 6-OHDA, yohimbine and WAY100635 were based on several studies that have investigated the pharmacological effects of the catecholaminergic neurotoxin, the α_2-adrenoceptor antagonist, and the 5-HT_{1A} receptor antagonist (Tanabe *et al.*, 2005; 2007). Yohimbine and WAY100635 were administered through the i.t. route 15 min before the formalin test. The formalin test was performed 3 days after the last treatment with either 6-OHDA or vehicle. For the i.t. injections, the drugs were given in a volume of 5 μL through a disposable 27-gauge needle, which was inserted into the subarachnoid space through the intervertebral foramen between L5 and L6 according to a method described by Hylden and Wilcox (1980). To deplete central 5-HT, DL-p-chlorophenylalanine (PCPA; Nacalai Tesque, Inc.) (300 $mg \cdot kg^{-1} \cdot day^{-1}$) was suspended in a 0.5% carboxymethylcellulose (Nacalai Tesque) sodium solution and administered i.p. for 5 consecutive days. The formalin test was conducted 1 day after the last treatment with either PCPA or vehicle (Tanabe *et al.*, 2007).

Mice were injected, in the rostral ventromedial medulla (RVM) or the locus coeruleus (LC), with GW9508 (10 ng per mouse) dissolved in saline. The intra-LC or RVM administration volume was 0.2 μL per mouse (GW9508). Briefly, mice were anaesthetized with pentobarbital (40 $mg \cdot kg^{-1}$) and immobilized on a stereotaxic surgery instrument (SR-5M; NARISHIGE Co., Ltd., Tokyo, Japan). A microsyringe with a 30 gauge stainless-steel needle was used for all experiments. The needle was inserted unilaterally into the LC (5.4 mm posterior to the bregma, 0.9 mm lateral from the midline and 4.75 mm deep) and the RVM (6.0 mm posterior to the bregma, 0 mm lateral from the midline and 6.5 mm deep). GW9508 (0.2 μL) were injected into the LC or RVM incrementally over the course of 1 min. The needle was kept at this position for 1 min after injection and then raised 1 mm. After another 30 s, the needle was slowly removed over a period of 1 min. The injection site of LC or RVM was confirmed with 0.5% Trypan blue in saline (0.2 μL per mouse).

Brain tissue preparation

As described in our previous study (Nakamoto *et al.*, 2012a), mice were deeply anaesthetized with sodium pentobarbital (65 $mg \cdot kg^{-1}$) and perfused transcardially with PBS, pH 7.4, which was followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4, 90 min following the termination of the GW9508 (n = 5) or 0.2% DMSO (n = 5) injections. Brain sections were collected, postfixed in 4% paraformaldehyde for 3 h, cryoprotected in 10% sucrose at 4°C for 3 h, and then placed in 20% sucrose at 4°C overnight. The following day, the tissues were frozen in optimal cutting temperature compound (Tissue-Tek OCT Compound, Sakura Finetek Japan, Co., Ltd., Tokyo, Japan) and stored at –80°C until use. Sections (15 μm thick) were cut with a cryostat (CM1850, Leica Microsystems GmbH, Wetzlar, Germany) and mounted on a MAS-coated glass slide (S9115, Matsunami Glass Ind., Ltd., Osaka, Japan). The delimitation of the RVM and LC was performed according to a stereotaxic atlas (Franklin and Paxinos, 2008).

Double immunofluorescence labelling

Immunohistochemical examinations were performed according to methods described previously (Nakamoto *et al.*, 2013). The brain sections were washed with PBS containing 0.1% TWEEN 20 (PBST) three times at 5 min intervals and incubated with blocking buffer (3% BSA in PBS and 5% normal donkey serum in 3% Triton X-100 in PBS) for 2 h at room temperature. The sections were then incubated with specific antibodies against the FFA1 receptor (rabbit polyclonal anti-GPR40/FFA1 recptor; 1:200; Abcam plc, Cambridge, MA, USA), NeuN (monoclonal anti-mouse NeuN; 1:1000; EMD Millipore Corporation, Billerica, MA, USA), glial fibrillary acidic protein (GFAP, mouse monoclonal anti-GFAP; 1:1000; EMD Millipore Corporation), tyrosine hydroxylase (TH; chicken polyclonal anti-TH; 1:200, Abcam plc), tryptophan hydroxylase (TPH; sheep polyclonal anti-TPH; 1:300; EMD Millipore Corporation), dopamine β-hydroxylase (DBH; goat polyclonal anti-DBH; 1:100; Abcam plc) or c-Fos (donkey polyclonal anti-Rabbit c-Fos; 1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), which were diluted in reaction buffer (1% BSA in PBS), for 48 h at 4°C. Sections were then washed with PBST six times for 5 min each and incubated in secondary antibodies that were conjugated with Alexa Fluor 488 and/or 594 (donkey polyclonal anti-rabbit IgG, goat polyclonal anti-mouse IgG, donkey polyclonal anti-goat IgG or donkey polyclonal anti-sheep IgG; 1:200; Life Technologies Corporation, Grand Island, NY, USA) and diluted in reaction buffer at room temperature for 2 h.

Finally, the sections were washed with PBST six times for 5 min each and coverslipped with PermaFluor (Thermo Fisher Scientific Inc., Waltham, MA, USA). Immunoreactivity was detected with a confocal fluorescence microscope (FV1000, Olympus Corporation, Tokyo, Japan). In control sections, no staining was detected when the corresponding primary or secondary antibody was omitted.

Quantitation of c-Fos immunoreactivity

For the quantitation of c-Fos immunoreactivity, mice were killed 90 min after the i.c.v., intra-LC and intra-RVM administration of GW9508 (1.0 µg or 10 ng) or 0.2% DMSO, and sections were viewed with a confocal fluorescence microscope (FV1000, Olympus Corporation). The coordinates of LC, which were bregma –5.4 mm, and the coordinates for the RVM, which were bregma –6.0 mm, were determined at the same level in each area according to a Mouse Brain Atlas (Franklin and Paxinos, 2008). The area of the RVM or the LC in the medulla oblongata was outlined, and the number of c-Fos-immunoreactive cells or double-positive cells (TPH^+/c-Fos^+ or DBH^+/c-Fos^+) in this area was counted. The analyses were performed by an observer who was unaware of the treatment of the animals. For the quantitative analysis of the c-Fos, TPH and DBH staining intensity, five brain sections were immunostained with each marker. For the LC and RVM, the number of c-Fos-positive cells for each section was determined from 500 × 500 μm^2 area, and then the average count (five sections) for each treated subject was calculated.

Formalin test

The formalin test was conducted as previously described (Hunskaar *et al.*, 1985). Briefly, mice were injected with 10 µL of 5% formalin [formaldehyde solution, 37% (w/w), diluted in saline] into the subplantar space of the right hind paw 10 min after i.c.v. drug administration. The time spent by the animal performing licking, biting and shaking behaviours was measured between 0 and 10 min (early phase) and between 10 and 45 min (late phase) after the formalin injection.

Extraction of 5-HT and noradrenaline from the mouse spinal cord

The extraction of 5-HT and noradrenaline from the spinal cord was performed according to methods described previously (Tsunoda *et al.*, 2011). We used a sample that included the spinal cords from three mice. The weight of the tissues was determined, and 1 mL of 0.05% Triton X/10 mM ammonium formate buffer (pH 4.0) was added per 0.1 g of wet tissue. The tissues were homogenized, and an equal volume of ice-cold chloroform/methanol (2:1, v/v) was added to the tissue homogenate. The mixture was centrifuged at 1300× *g* for 30 min at 4°C. The upper phase (the buffer phase) was removed and centrifuged at 15 000× *g* for 30 min at 4°C. Finally, 5-HT and noradrenaline were extracted from the supernatant with a Monospin PBA column (GL Sciences Inc., Tokyo, Japan).

LC-MS/MS analysis of monoamines in the mouse spinal cord

The LC-MS/MS analysis was performed according to previously described methods (Tsunoda *et al.*, 2011). High-performance LC separation was performed with a Waters 2690 instrument (Waters Corporation, Milford, MA, USA) with a COSMOSIL PBr column: 2.0 mm internal diameter × 150 mm (Nakalai Tesque Inc.). The mobile phases were 10 mM ammonium formate [(pH 4.0), A] and acetonitrile (B). The eluting gradient was as follows: the column was equilibrated with 100% A and eluted with 100% A for 3 min, 100% A to 40% A for 12 min and 40% A to 100% A for 10 min. The flow rate was 0.2 $mL \cdot min^{-1}$. Quantitation was conducted with a Quattro Ultima (Waters Corporation). The mass spectrometer was operated in the positive-ion mode with the source temperature set to 100°C, a cone voltage of 20 V and a capillary voltage of 3.5 kV. The collision energy was individually tuned at 20 eV. All positive-ion mass spectral data were obtained by scanning the mass range from m/z 80 to 200. 5-HT and noradrenaline were quantified by selective multi-reaction monitoring with a positive ionization mode. The peak of each of the catecholamines was monitored by the product ion obtained from [M + H] + ion (i.e., m/z 154 → m/z 91 for dopamine, m/z 169.9 → m/z 107 for noradrenaline and m/z 177 → m/z 115.1 for 5-HT; Tsunoda *et al.*, 2011). The increment ratio of 5-HT or noradrenaline after the GW9508 injection was expressed as the percentage of the content of vehicle group.

Data analysis

The data are expressed as the mean ± SEM. The data were analysed with GraphPad prism version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between two groups were evaluated by Student's *t*-tests. Statistical analyses of the time course data for the formalin test were conducted with two-way repeated measures analysis of variance (ANOVA), which was followed by a *post hoc* Bonferroni's comparison test. Fisher's exact test was used to analyse two categorical variables. *P*-values less than 0.05 were considered significant.

Results

Expression of FFA1 receptors in the medulla oblongata

FFA1 receptors (green) were expressed in the RVM (Figure 1A) and the LC (Figure 1B) of the medulla oblongata. Similar to these receptors (green), immunoreactivity for NeuN (a neuron marker; red) or GFAP (an astrocyte marker; red) was also observed in the RVM (Figure 1A) and LC (Figure 1B) of the medulla oblongata. To investigate the precise localization of FFA1 receptors, double immunostaining with FFA1 receptor and anti-NeuN and anti-GFAP antibodies was performed. The FFA1 receptors were colocalized with NeuN but not with GFAP in the RVM (Figure 1A) and LC (Figure 1B) of the medulla oblongata.

FFA1 receptor colocalization with TPH or DBH in the medulla oblongata

Immunoreactivity for TPH (a serotonergic neuron marker) and DBH (a noradrenergic neuron marker) was observed in the RVM and LC of the medulla oblongata respectively. To determine which neurons localized with FFA1 receptors, double immunostaining with FFA1 receptor and TPH and

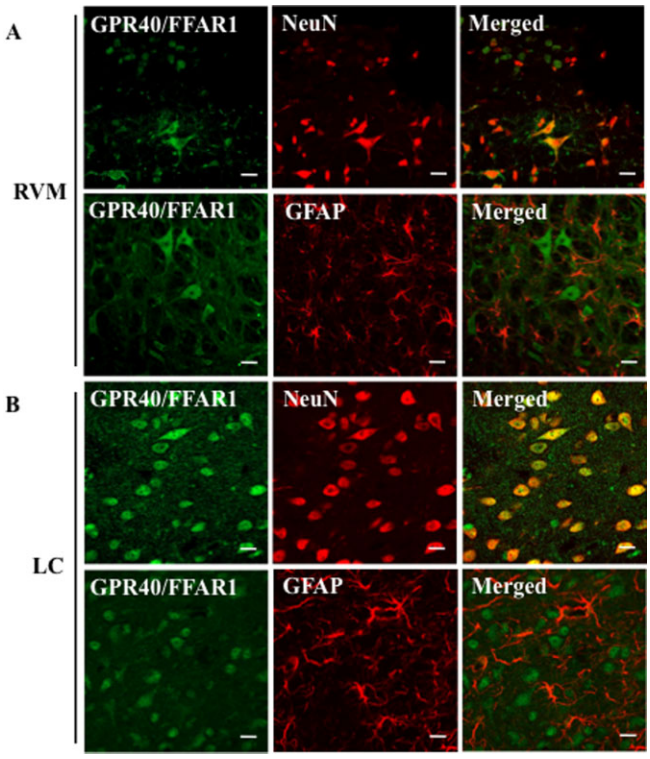

Figure 1

Confocal microscopic images of neurons double-labelled with the FFA1 receptor (GPR40/FFAR1) and neuron or astrocyte markers in the RVM (A) and LC (B) of the medulla oblongata. Colocalization of FFA1 receptors with NeuN (a neuronal marker) or GFAP (an astrocytic marker) in the medulla oblongata was evaluated with double immunofluorescence staining (green: FFA1 receptors ; red: NeuN or GFAP). Representative images are shown. Scale bars: 10 μm (original magnification 100×).

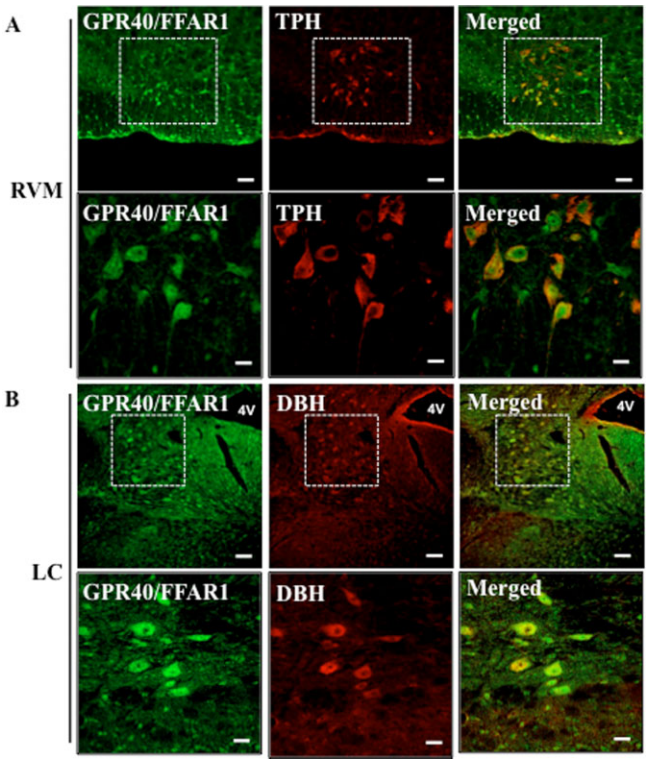

Figure 2

Colocalization of FFA1 receptors (GPR40/FFAR1) with TPH (a serotonergic neuron marker) and DBH (a noradrenergic neuron marker) in the brain. Colocalization in the RVM (A) and the LC (B) was evaluated with double immunofluorescence staining (green: FFA1 receptors; red: TPH or DBH). Representative images are shown. Scale bars: 50 μm (original magnification 20×, upper panel A, B), 50 μm (original magnification 100×, lower panel A, B).

DBH antibodies was performed. The FFA1 receptors were colocalized with TPH in the RVM (Figure 2A) and with DBH in the LC of the medulla oblongata (Figure 2B).

GW9508-induced c-Fos expression and increased colocalization with TPH in the RVM

We examined whether a FFA1 receptor agonist activated TPH-positive neurons in the RVM. The expression of c-Fos in the RVM of the medulla oblongata was induced by a single i.c.v. injection of GW9508 (1.0 μg) or 0.2% DMSO (Figure 3A). This induction was found in TPH-positive neurons (Figure 3A and B). With i.c.v. injection of GW9508 (1.0 μg), the number of c-Fos-positive cells in the RVM, including the raphe nuclei of the medulla oblongata, was significantly increased compared with that in the 0.2% DMSO-treated group (Figure 3C, $P < 0.05$). Furthermore, the number of neurons that were double-labelled for c-Fos and TPH increased in the mice treated with GW9508 compared with that in the 0.2% DMSO-treated group (Figure 3D, $P < 0.05$). To confirm whether or not FFA1 receptors in the RVM directly contributed to the activation of descending pain control system, we examined the effects of microinjection of GW9508 into the RVM. The microinjection of GW9508 (10 ng) into the RVM induced c-Fos expression in the RVM compared with that in the 0.2% DMSO-treated group (Figure 3E and F).

GW9508-induced c-Fos expression and increased colocalization with DBH in the LC

Next, we examined whether the FFA1 receptor agonist activated DBH-positive neurons in the LC. The expression of c-Fos in the LC was induced by a single i.c.v. injection of GW9508 (1.0 μg) or 0.2% DMSO (Figure 4A). This induction was found in DBH-positive neurons (Figure 4A and B). With the i.c.v. injection of GW9508 (1.0 μg), the number of c-Fos-positive cells in the LC was significantly increased compared with that in the 0.2% DMSO-treated group (Figure 4C, $P < 0.05$). Furthermore, there was an increase in the number of neurons that were double-labelled for c-Fos and DBH in the mice treated with GW9508 compared with that in the 0.2% DMSO-treated group (Figure 4D, $P < 0.05$). To confirm whether or not FFA1 receptors in the LC directly contributed to the activation of descending pain control system, we examined the effects of microinjection of GW9508 into the LC. The microinjection of GW9508 (10 ng) into the LC increased c-Fos expression in the LC, compared with that in the 0.2% DMSO-treated group (Figure 4E and F).

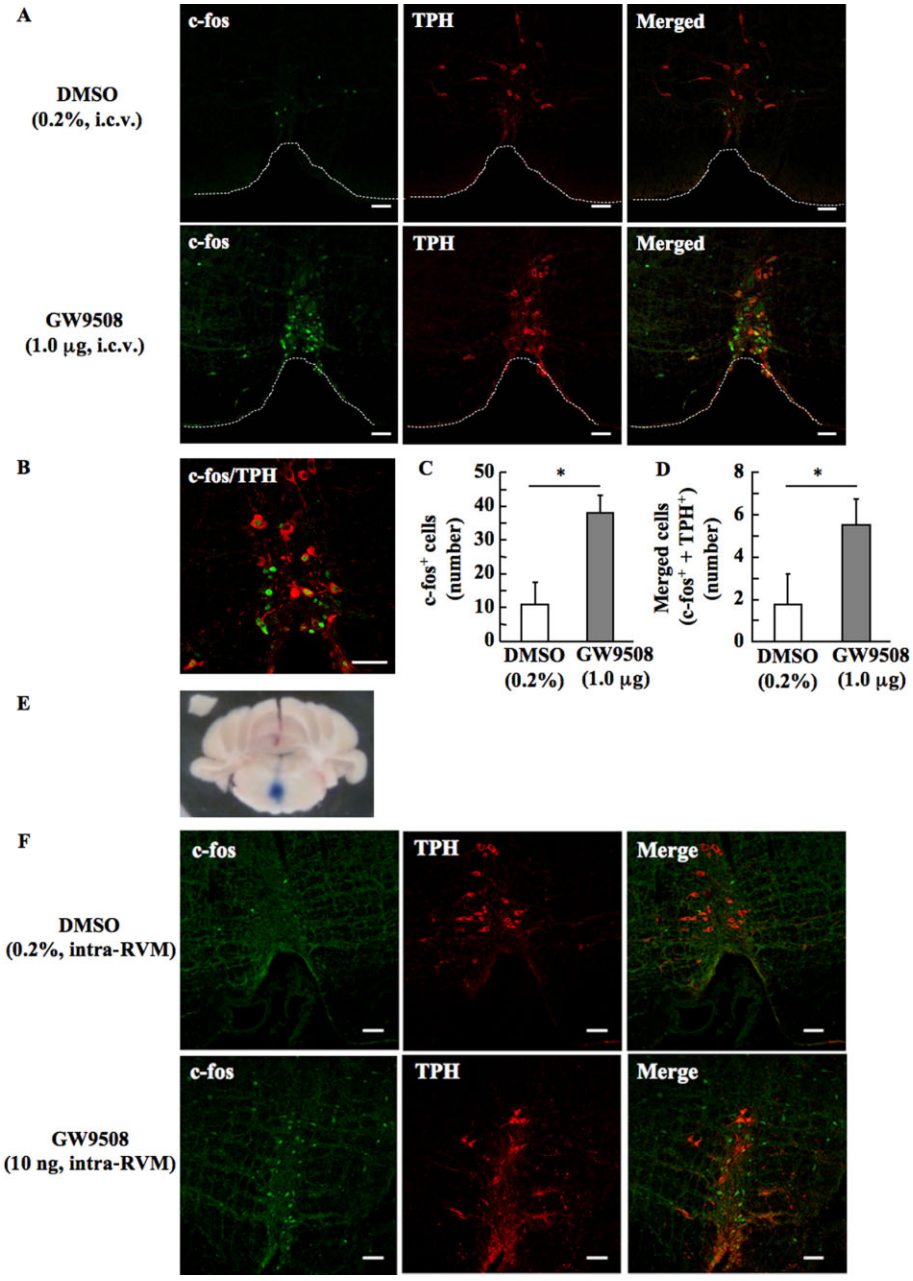

Figure 3

Influence of GW9508 on the induction of c-Fos protein in the RVM. Colocalization of c-Fos with TPH in the RVM after the i.c.v. administration of GW9508 (1.0 μg) or vehicle was evaluated with double immunofluorescence staining (green: c-Fos; red: TPH) (A). The image shows a high magnification of the colocalization of neurons that were double-labelled with TPH and c-Fos in the RVM (green: c-Fos; red: TPH) (B). Summaries of the data on the number of c-Fos cells (C) and merged cells (c-Fos$^+$/TPH$^+$) (D) are shown on the bottom; data, mean ± SEM. The injection site of RVM was confirmed with 0.5% Trypan blue in saline (0.2 μL per mouse) (E). Colocalization of c-Fos with TPH in the RVM after the intra-RVM administration of GW9508 (10 ng) or vehicle was evaluated with double immunofluorescence staining (green: c-Fos; red: TPH) (F); 0.2% DMSO; (n = 5), 1.0 μg GW9508 (n = 5); *P < 0.05 compared with 0.2% DMSO, Student's t-test. Representative images are shown. Scale bars: 50 μm (original magnification 20×, A and F), 10 μm (original magnification 100×, B).

GW9508-induced antinociception was inhibited by PCPA pretreatment

Subcutaneous injections of formalin (10 μL, 5%) into the hind paw caused a biphasic response of licking or biting of the injected paw. The first phase started immediately after the injection and lasted for about 5 min, while the second phase began 10 min after the injection and lasted for approximately 45 min (Figure 5A). To examine the influence of descending serotonergic neurons on GW9508-induced antinociception, PCPA was administered i.p. for 5 consecutive days. The formalin test was performed 1 day after the last treatment with either

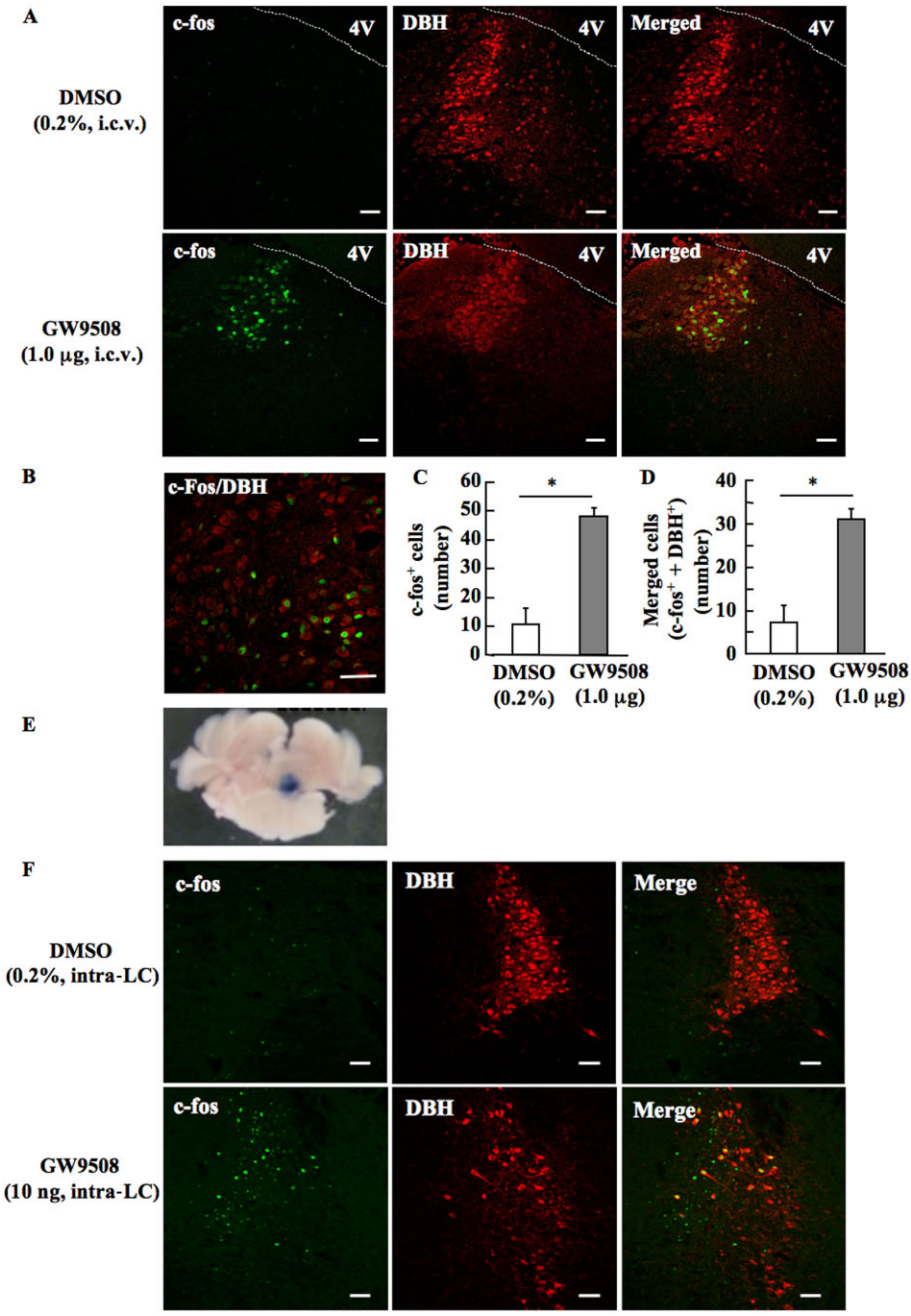

Figure 4

Influence of GW9508 administration on the induction of c-Fos protein in the LC. Colocalization of c-Fos with DBH in the LC was evaluated with double immunofluorescence staining (green: c-Fos; red: DBH) (A). The image shows a high magnification of the colocalization of neurons that were double-labelled with DBH and c-Fos in the LC (B) (green: c-Fos; red: DBH). Summaries of the data on the number of c-Fos cells (C) and merged cells (c-Fos$^+$/DBH$^+$) (D) are shown on the bottom; data, mean ± SEM. The injection site of LC was confirmed with 0.5% trypan blue in saline (0.2 μL per mouse) (E). Colocalization of c-Fos with TPH in the LC after the intra-LC (10 ng) administration of GW9508 or vehicle was evaluated with double immunofluorescence staining (green: c-Fos; red: DBH) (F); 0.2% DMSO (n = 5), 1.0 μg GW9508 (n = 5); *P < 0.05 compared with 0.2% DMSO, Student's t-test. Representative images are shown. Scale bars: 50 μm (original magnification 20×, A and F), 10 μm (original magnification 100×, B).

PCPA or vehicle (Figure 5A). The spinal and brain 5-HT contents were significantly suppressed by repeated administration of PCPA (Figure 5B, P < 0.01). The i.c.v. injection of GW9508 (1.0 μg) significantly decreased the formalin-induced pain-related behaviour in the late phase (10–45 min; P < 0.05) but not in the early phase (0–10 min) compared with that in the vehicle-treated group. This effect of GW9508 was significantly inhibited by i.p. PCPA pretreatment (Figure 5C; P < 0.05).

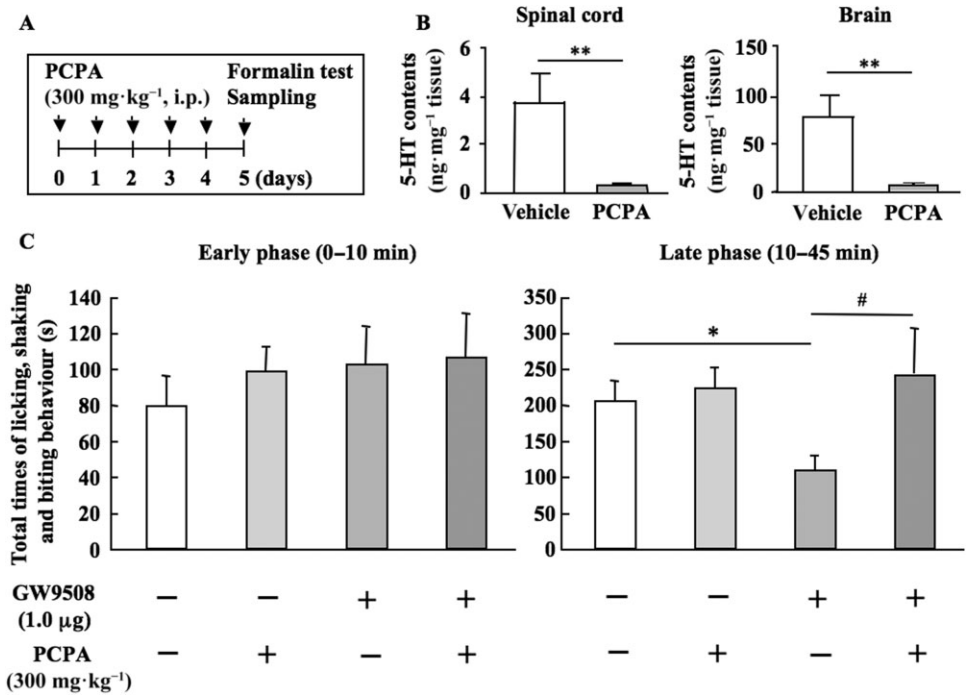

Figure 5

Effects of PCPA on GW9508-induced antinociception. The cumulative nociceptive response time consisting of biting, licking and shaking of the injected hind paw was measured during a 45 min period after injection (intraplantar, i.pl.) of formalin at 5% (v/v). Responses were measured at 0–10 min (early phase) and 10–45 min (late phase) after the formalin injection. PCPA (300 mg·kg^{-1}·day^{-1}) was administered i.p. for 5 consecutive days (A and C). The spinal and brain serotonin contents after repeated administration of PCPA (B). Each column indicates the mean ± SEM (n = 8). $^{**}P < 0.01$ compared with vehicle, Student's t-test. $^{*}P < 0.05$ vs. vehicle, $^{\#}P < 0.05$ vs. GW9508 (two-way repeated measures ANOVA, with *post hoc* Bonferroni's comparison test).

GW9508-induced antinociception was inhibited by 6-OHDA pretreatment

To examine the influence of descending noradrenergic neurons on GW9508-induced antinociception, the formalin test was performed 3 days after the last treatment with either 6-OHDA (20 μg) or vehicle (Figure 6A). Pretreatment with 6-OHDA (20 μg) suppressed DBH-positive neurons in the LC (Figure 6B). GW9508-induced antinociception was significantly inhibited by 6-OHDA (i.t.; 20 μg) pretreatment (Figure 6C; $P < 0.05$).

Yohimbine or WAY100635 inhibited GW9508-induced antinociception

The i.t. injection of yohimbine (4 μg) significantly inhibited GW9508-induced antinociception in the late phase (10–45 min) of formalin-induced pain behaviour ($P < 0.01$) but not in the early phase (0–10 min; Figure 7). The i.t. injection of WAY100635 (10 μg) also significantly inhibited GW9508-induced antinociception in the late phase (10–45 min; $P < 0.01$) but not in the early phase (0–10 min) compared with that in the GW9508-treated group (Figure 7). Yohimbine or WAY100635 given alone, had no effects on the formalin-induced pain behaviour.

GW9508 increased noradrenaline and 5-HT in the spinal cord

To confirm whether an i.c.v. injection of GW9508 activated the descending serotonergic and noradrenergic neurons, 5-HT and noradrenaline release in the spinal cord was analysed with LC-MS/MS. In the intact mice, i.c.v. injections of GW9508 had no effect on 5-HT and noradrenaline levels in the spinal cord (Figure 8A and B). On the other hand, in formalin-injected mice, the increment ratio of spinal 5-HT showed a tendency to increase (Figure 8C, $P = 0.0689$), and the increment ratio of spinal noradrenaline levels was significantly increased after GW9508 administration in formalin-injected mice compared with that in the saline-treated group (Figure 8D, $P < 0.01$).

GW1100 exacerbated formalin-induced pain behaviour

Finally, to confirm whether supraspinal FFA1 receptor signalling was related to the regulation of descending serotonergic and noradrenergic neurons, GW1100, which is a FFA1 receptor antagonist, was used in the formalin test. An i.c.v. injection of GW1100 (10 μg) significantly increased the total times of formalin-induced pain behaviour in the late phase (10–45 min; $P < 0.01$) but not in the early phase (0–10 min) compared with that in the vehicle-treated group (Figure 9A and B).

Discussion

The descending noradrenergic LC regions, as well as RVM systems, which are related to serotonergic neurons, project

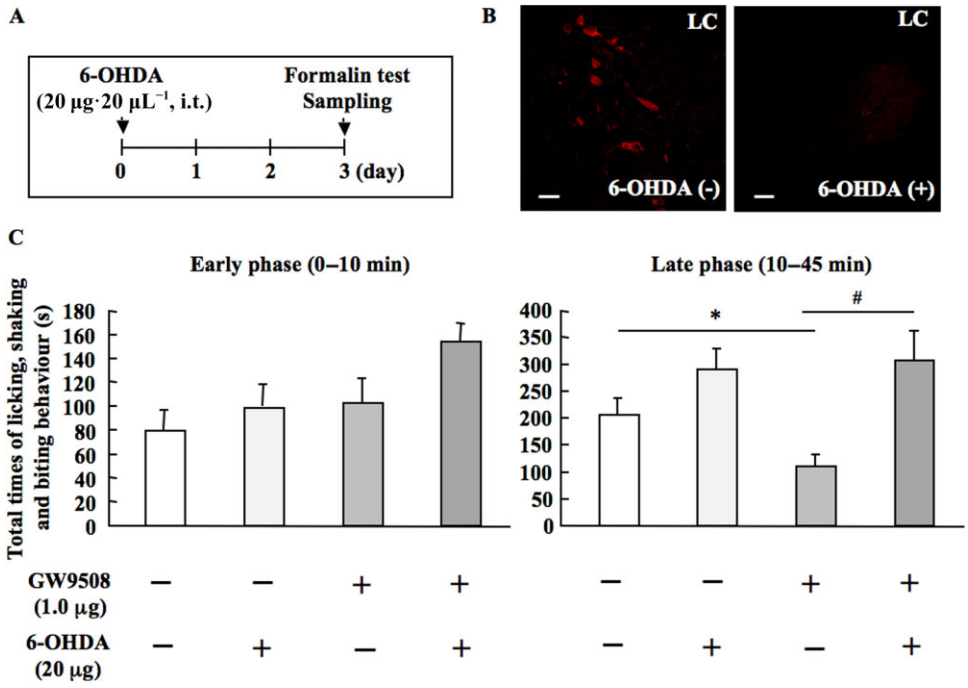

Figure 6

Effects of 6-OHDA on GW9508-induced antinociception. The cumulative nociceptive response time consisting of biting, licking and shaking of the injected hind paw was measured during a 45 min period after the injection (i.pl.) of 5% (v/v) formalin. The responses were measured at 0–10 min (early phase) and 10–45 min (late phase) after the formalin injection. Pretreatment with 6-OHDA (20 μg) was performed by i.t. route 3 days before the formalin test (A and C). Immunohistochemical analysis of TH in the LC of vehicle or 6-OHDA-injected mice (B). Representative images are shown. Scale bars: 10 μm. Each column indicates the mean ± SEM ($n = 8$). *$P < 0.05$ vs. vehicle, #$P < 0.05$ vs. GW9508 (two-way repeated measures ANOVA with *post hoc* Bonferroni's comparison test).

their axons to the spinal cord (Vanegas and Schaible, 2004). An impairment of descending inhibition and an enhancement of facilitation during chronic pain situations increase nociceptive transmission at the spinal cord (Tracey and Mantyh, 2007). Therefore, it is thought that the regulation of descending inhibitory systems plays an important role in the pathology of pain. In the present study, we focused on FFA1 receptor signalling in the medulla oblongata and found that these receptors were expressed on serotonergic neurons in the RVM, including the raphe nuclei of the medulla oblongata, and on noradrenergic neurons in the LC. These findings supported our previous study that FFA1 receptors were expressed on neurons but not on astrocytes (Nakamoto *et al.*, 2013).

In addition, we examined the expression of c-Fos, which is a marker of activated neurons, with a double immunohistochemical study in order to clarify whether the i.c.v. administration of a FFA1 receptor agonist induced neuronal activity in the RVM and LC. It is well known that c-Fos is rapidly but transiently expressed following cellular stimulation and that c-Fos levels reflect stimulus-induced neuronal activation at the cellular level (Sagar *et al.*, 1988). In this study, a marked induction of c-Fos in the RVM and LC was observed, and the number of double-positive neurons (c-Fos$^+$/TPH$^+$ or c-Fos$^+$/DBH$^+$) was also increased by the i.c.v. injection of GW9508, indicating that injections of the FFA1 receptor agonist may activate these neurons and produce antinociception. Furthermore, we found that the microinjection of GW9508 into the LC or the RVM induced c-fos expression in the cell body of this descending neuron. These results indicate that the FFA1 receptor signalling system in the LC or RVM might directly activate the descending noradrenergic or serotonergic neurons. Based on our results and our previous reports, it is thought that FFA1 receptor signalling in the brain may directly or indirectly activate the descending pain control system. However, the numbers of the merged cells, especially c-fos and TPH double-positive cells, were fewer than that of c-fos-positive cells. Generally, neurons in the RVM are involved in descending modulation of nociceptive transmission in the spinal cord (Ossipov *et al.*, 2010; Khasabov and Simone, 2013). RVM neurons are two distinct population 'on-cells' and 'off-cells' (Fields *et al.*, 1983). Both the off-cells and on-cells were found to project to the spinal dorsal horn, indicating that they may exert modulatory influences on nociceptive inputs (Fields *et al.*, 1995). In the RVM area, TPH, GABA or glycine-positive cells are present and many cell bodies are found in the RVM, and thus might contribute to the regulation of anti- or pro-nociceptive effects. For example, neuroanatomical studies reported a high degree of colocalization of cholecystokinin 2 and opioid receptors on RVM neurons, presumed to be pain μ-opioid receptors with facilitation cells that may correspond with on-cells (Zhang *et al.*, 2009). And also, neurokinin-1 receptors, which are presumably located on pain facilitating on-cells, are found in the RVM. Furthermore, most off-cells, on-cells and neutral cells have been shown to express glutamate decarboxylase

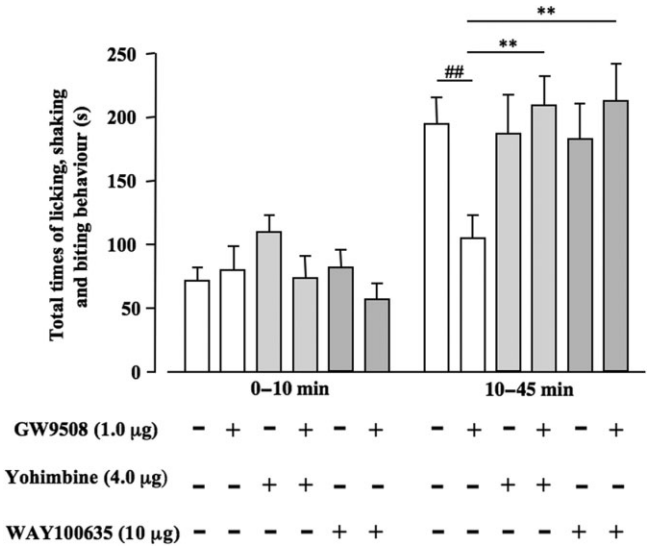

Figure 7

Effects of yohimbine or WAY100635 on GW9508-induced antinociception. The cumulative nociceptive response time consisting of biting, licking and shaking of the injected hind paw was measured during a 45 min period after injections (i.pl.) of 5% (v/v) formalin. Responses were measured at 0–10 min (early phase) and 10–45 min (late phase) after the formalin injection. Pretreatment with yohimbine (4 μg) or WAY100635 (10 μg) was performed by i.t. route 15 min before the formalin test. Each column indicates the mean ± SEM ($n = 8$). $^{\#\#}P < 0.01$ vs. vehicle, $^{**}P < 0.01$ vs. GW9508 (two-way repeated measures ANOVA with *post hoc* Bonferroni's comparison test).

(Winkler *et al.*, 2006). However, the role of GABA in the function of these cells remains unclear. In this study, we showed that FFA1 receptors were present in the RVM, and that the i.c.v. or intra-RVM administration of the corresponding receptor agonist activated these neurons. Therefore, we have concluded that FFA1 receptor agonists, in part, might activate serotonergic neurons in the RVM. However, further studies will be needed to clarify the cell types activated by the administration of FFA1 receptor agonists.

To test whether FFA1 receptor-induced antinociception was mediated through the descending pain control system, we used a neuronal toxin to deplete 5-HT and noradrenaline and antagonists to block 5-HT_{1A} receptors and/or α_2-adrenoceptors. It has been reported that the noradrenergic pain inhibitory system and the α_2-adrenoceptors in the lumbar spinal cord are sequentially activated to generate analgesic effects (Tanabe *et al.*, 2005). Intrathecally applied α_2-adrenoceptor agonists exert analgesic effects on both acute thermal (Hunter *et al.*, 1997; Stone *et al.*, 1997) and mechanical nociception (Ochi and Goto, 2000). Moreover, the electrical stimulation of noradrenergic nuclei in the brainstem can generate spinal α_2-adrenoceptor-mediated analgesic effects on acute thermal nociception (Jones and Gebhart, 1986). Hence, the descending noradrenergic system coupled with spinal α_2-adrenoceptors can potentially influence acute nociception. In contrast, the descending serotonergic pathways have been demonstrated to exert both pro- and anti-nociceptive actions. Anti-nociception through serotonergic neurons may involve several classes of 5-HT receptors that are activated by released 5-HT in the spinal cord (Millan, 2002). Of all the classes of 5-HT receptors, 5-HT_{1A} receptors in the spinal cord have been clearly shown to participate in the modulation of nociception (Bardin, 2011).

We initially expected that either noradrenergic or serotonergic neurons were the main target in GW9508-induced antinociception. In this study, however, GW9508-induced antinociception was inhibited by pretreatment with a catecholaminergic neurotoxin, a 5-HT synthesis inhibitor, and each receptor antagonist related to the descending pain control system. Furthermore, i.c.v. injections of GW9508 increased noradrenaline and 5-HT in the spinal cord of formalin-injected mice, whereas the i.c.v. injections of GW9508 without formalin injection had no effect on the level of spinal noradrenaline and 5-HT. Clearly GW9508 was more effective after a nociceptive stimulus, such as formalin. A possible mechanism underlying this increased effectiveness is that the supraspinal FFA1 receptor signalling system may be more active during pain and this would allow the exogenous agonist GW9508 to induce a greater release of neurotransmitters (noradrenaline and 5-HT), than in the absence of the nociceptive stimulus. Our results suggested that the GW9508-induced activation of brain FFA1 receptor signalling may act on both types of neurons and may produce antinociception through the activation of the descending pain control systems.

In our previous study, increasing levels of free fatty acids were observed in the hypothalamus during inflammatory pain induced by complete Freund's adjuvant, suggesting that free fatty acids may be continuously released during pain (Nakamoto *et al.*, 2013). Therefore, it is possible that the FFA1 receptor that is expressed on these neurons is activated by long-chain fatty acids, indicating that FFA1 receptor signalling may contribute to pain control. Hence, we hypothesized that the activation of FFA1 receptor signalling that occurs through increases of endogenous PUFA in both noradrenaline and serotonin neurons may help in the suppression of pain behaviour. To clarify this hypothesis, we examined whether pretreatment with GW1100 exacerbated formalin-induced pain behaviour. In the late phase of formalin-induced pain, i.c.v. pretreatment with GW1100 significantly exacerbated formalin-induced pain-like behaviour. Overall, our results would suggest that, following a nociceptive stimulus, FFA1 receptor signalling, which is activated by endogenous free fatty acids, may play a key role in the regulation of the endogenous pain control system, which is comprised of serotonergic and noradrenergic neurons.

In summary, in the present study, we demonstrated that FFA1 receptors were expressed by serotonergic and noradrenergic neurons. In addition, these descending neurons were directly or indirectly activated by the administration of a FFA1 receptor agonist. Furthermore, a FFA1 receptor antagonist exacerbated formalin-induced pain. We suggest that the supraspinal FFA1 receptor signalling system plays an important role in the regulation of the descending pain control system. Our findings provide insight into the mechanisms underlying intractable pain and may help in the development of therapeutic strategies for such conditions.

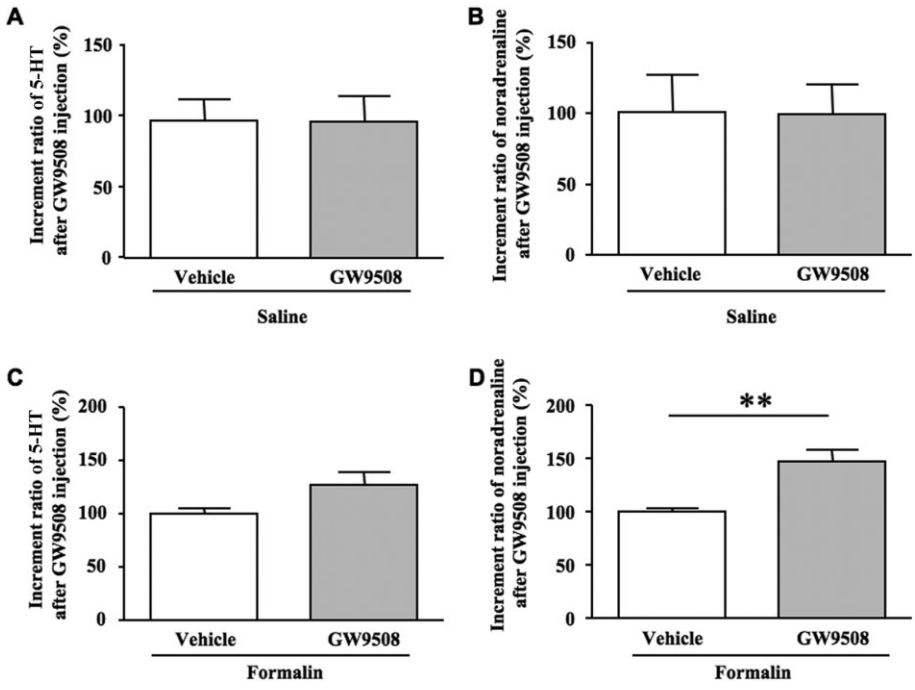

Figure 8

The quantitative analysis of noradrenaline and 5-HT in the spinal cord after the i.c.v. administration of GW9508 or vehicle in saline (A and B) or formalin (C and D) injected mice. 5-HT (A and C) and noradrenaline (B and D) were extracted from the spinal cord, and their levels were analysed by LC-MS/MS. The analyses were performed on samples collected from the spinal cord of three mice. Each column indicates the mean ± SEM (n = 9). $^{**}P < 0.01$ compared with vehicle, Student's t-test.

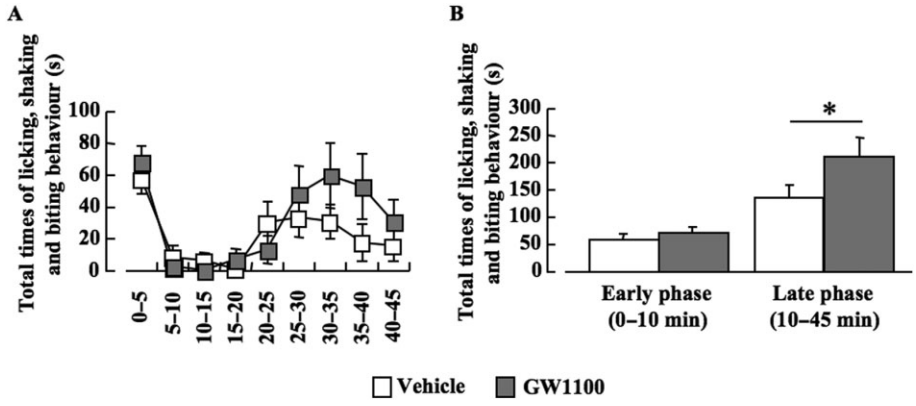

Figure 9

Effects of GW1100 on formalin-induced pain behaviour. The cumulative nociceptive response time consisting of biting, licking and shaking of the injected hind paw was measured during a 45 min period after the injection (i.pl.) of 5% (v/v) formalin. The responses were measured at 0–10 min (early phase) and 10–45 min (late phase) after the formalin injection. GW1100 was administered by the i.c.v. route 10 min before the formalin test. (A) Time course of pain behaviour after the i.pl. injection of formalin with or without GW1100. (B) Antinociceptive effect of GW1100 on formalin test. Each column indicates the mean ± SEM (n = 8). $^{*}P < 0.05$ vs. vehicle (two-way repeated measures ANOVA with *post hoc* Bonferroni's comparison test).

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Author contributions

Study conception and design: K. N. and S. T. Acquisition of data: K. N., T. N., N. S. and F. A. Analysis and interpretation: K. N., Y. K., F. K., M. M. and S. T. Drafting of manuscript: K. N. and S. T. Critical revision: S. T.

Conflict of interest

None.

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