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RESEARCH PAPER

# Nociceptin/orphanin FQ peptide receptor antagonist JTC-801 reverses pain and anxiety symptoms in a rat model of post-traumatic stress disorder

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## BACKGROUND AND PURPOSE

Single-prolonged stress (SPS), a rat model of post-traumatic stress disorder (PTSD), also induces long-lasting hyperalgesia associated with hypocortisolism and elevated nociceptin/orphanin FQ (N/OFQ) levels in serum and CSF. Here, we determined the effect of JTC-801 (N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide monohydrochloride), a nociceptin/orphanin FQ peptide (NOP) receptor antagonist, on symptoms of pain and anxiety in rats after SPS exposure, and examined N/OFQ-NOP receptor system changes.

## EXPERIMENTAL APPROACH

Male Sprague Dawley rats received JTC-801 (6 mg $kg^{-1}$ i.p., once daily) during days 7–21 of SPS. The ability of JTC-801 to inhibit N/OFQ-stimulated [$^{35}$S]-GTPγS binding was confirmed in rat brain membranes. Anxiety-like behaviour and pain sensitivity were monitored by changes in elevated plus maze performance and withdrawal responses to thermal and mechanical stimuli. Serum corticosterone and N/OFQ content in CSF, serum and brain tissues were determined by radioimmunoassay; NOP receptor protein and gene expression in amygdala, hippocampus and periaqueductal grey (PAG) were examined by immunoblotting and real-time PCR respectively.

## KEY RESULTS

JTC-801 treatment reversed SPS-induced mechanical allodynia, thermal hyperalgesia, anxiety-like behaviour and hypocortisolism. Elevated N/OFQ levels in serum, CSF, PAG and hippocampus at day 21 of SPS were blocked by JTC-801; daily JTC-801 treatment also reversed NOP receptor protein and mRNA up-regulation in amygdala and PAG.

## CONCLUSION AND IMPLICATIONS

JTC-801 reversed SPS-induced anxiety- and pain-like behaviours, and NOP receptor system up-regulation. These findings suggest that N/OFQ plays an important role in hyperalgesia and allodynia maintenance after SPS. NOP receptor antagonists may provide effective treatment for co-morbid PTSD and pain.

## LINKED ARTICLES

This article is part of a themed section on Opioids: New Pathways to Functional Selectivity. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-2

## Abbreviations

CORT, corticosterone; EPM, elevated plus maze; HPA, hypothalamic–pituitary–adrenal; JTC-801, N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide monohydrochloride; N/OFQ, nociceptin/orphanin FQ; PTSD, post-traumatic stress disorder; PAG, periaqueductal gray; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; RIA, radioimmunoassay; SPS, single-prolonged stress

## Introduction

Frequent co-occurrence of chronic pain and post-traumatic stress disorder (PTSD) has been well documented in the literature, as has the elevated prevalence of chronic pain in both civilian and veteran PTSD populations in the USA (Shipherd *et al.*, 2007; Asmundson and Katz, 2009; Lew *et al.*, 2009). However, current knowledge about the interaction between chronic pain and PTSD is still lacking, largely due to the difficulty of translation from animal models of PTSD to clinical studies. Single-prolonged stress (SPS), an established animal model for PTSD (Liberzon *et al.*, 1997; 1999), has received much attention in recent years. This model mimics many of the physiological and behavioural changes described in PTSD patients, including anxiety and fear behaviour, and enhanced negative feedback to the hypothalamic–pituitary–adrenal (HPA) axis (Yamamoto *et al.*, 2009). We have confirmed HPA axis dysfunction and anxiety symptoms of SPS and reported increased pain sensitivity in this model (Zhang *et al.*, 2012). Hyperalgesia to both thermal and mechanical stimuli emerged as early as 7 days after initiation of SPS, and was maintained for at least 28 days, suggesting that PTSD-like conditions decrease the pain threshold and exaggerate nociceptive sensitivity.

Nociceptin/orphanin FQ (N/OFQ), an endogenous ligand for the N/OFQ peptide (NOP) receptor, modulates nociceptive processing, HPA axis and stress, anxiety and immune responsiveness (Lambert, 2008; receptor nomenclature follows Alexander *et al.*, 2013); important processes affected by PTSD. This makes the NOP receptor a potentially important target for the treatment of chronic pain and PTSD. Numerous studies have revealed a role for N/OFQ in pain modulation. In contrast to its analgesic properties upon spinal administration (King *et al.*, 1997; Tian *et al.*, 1997; Yamamoto *et al.*, 1997), supraspinal administration of N/OFQ produces hyperalgesia in rats (Mogil *et al.*, 1996; Tian *et al.*, 1997; Pan *et al.*, 2000). Elevated N/OFQ levels in serum and CSF are associated with pain or pain control in human subjects (Ko *et al.*, 2002; Raffaeli *et al.*, 2006). CSF and/or serum levels of N/OFQ also are elevated in animal models of chronic neuropathic pain (Cornefjord *et al.*, 2004; Joseph *et al.*, 2007; Liu *et al.*, 2012). The NOP receptor antagonist, JTC-801 ((N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide monohydrochloride), a nociceptin), exerts antinociceptive actions towards both acute (Shinkai *et al.*, 2000; Yamada *et al.*, 2002) and chronic pain (neuropathic, Suyama *et al.*, 2003; Tamai *et al.*, 2005) when administered systemically. We have reported that SPS-induced persistent allodynia and hyperalgesia was accompanied by elevated levels of N/OFQ in serum and CSF (Zhang *et al.*, 2012). Therefore, we hypothesized that N/OFQ contributes to the maintenance of hyperalgesia and allodynia during chronic pain and PTSD, thus exacerbating both conditions. The current study sought to determine if JTC-801, by reducing N/OFQ-NOP receptor system activity, would alter nociceptive sensitivity and other PTSD symptoms in rats subjected to SPS. Our results would suggest that up-regulation of the N/OFQ-NOP receptor system contributes to increased pain sensitivity, hypocortisolism and anxiety symptoms in this animal model of PTSD.

## Methods

### Animals

All animal care complied with the Animal Welfare Act Regulations and other Federal Statutes relating to animals and experiments involving animals, and adhered to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center and the US Army Medical Research and Materiel Command Animal Care and Use Review Office. Experiments conformed to the guidelines of the International Association for the Study of Pain. Efforts were made throughout experiments to minimize animal discomfort and reduce the number of animals used. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 43 animals were used in the experiments described here.

Adult male Sprague Dawley rats, weighing 220–250 g at the initiation of SPS, were obtained from Charles River Labs (Wilmington, MA, USA). Animals were housed in the animal facility under a 12 h light : 12-h dark cycle (lights on at 06:00 h) with free access to food and water. After arrival, rats were acclimated to the animal facility for 7–10 days before experiments were initiated.

### SPS and drug treatment

Rats were randomly divided into vehicle (Veh; $n = 9$), JTC-801 ($n = 10$), SPS ($n = 12$) and SPS + JTC-801 ($n = 12$) groups. The SPS procedure was conducted as described (Liberzon *et al.*, 1997; 1999) with slight modification (Zhang *et al.*, 2012). After 7 days of acclimatization, rats were exposed to complete restraint in disposable plastic holders for 2 h, followed by grouped (3–4 rats) forced swimming for 20 min in a cylindrical Plexiglas tank (46 cm tall × 20 cm in diameter) filled with 22°C water to a depth of 30 cm. After 15 min recovery, rats were exposed to diethyl ether until loss of consciousness, and then left undisturbed and alone for 7 days. Since increased nociceptive sensitivity was maximal by day 9 and maintained through at least 28 days in our previous study, dose and length of treatment of JTC-801 with daily i.p. injection were determined by pilot and previous studies (Mabuchi *et al.*, 2003; Gunduz *et al.*, 2011): JTC-801 dose dependently (0.1–10 mg $kg^{-1}$, i.p.) produced anti-allodynic effects in both mechanical and cold tests in a mouse sciatic nerve injury model (Gunduz *et al.*, 2011), and a single dose (3 mg $kg^{-1}$, oral) increased withdrawal latency 7 days after spinal nerve ligation (Mabuchi *et al.*, 2003). We tested the efficacy of JTC-801 in rats after chronic constrictive injury (1 mg $kg^{-1}$, i.p., twice daily from days 1–12). JTC-801 alleviated mechanical and thermal hyperalgesia but was unable to completely reverse pain sensitivity to baseline. Two daily injections (3 mg $kg^{-1}$) reversed allodynia but induced inflammation in the injection site in a few rats; therefore, we settled on a single daily injection of 6 mg $kg^{-1}$. Beginning at day 7 after SPS, rats were injected with JTC-801 prepared in 3% DMSO and 0.05% hydroxypropylcellulose at dose of 6 mg $kg^{-1}$ once daily between 16:30 and 17:00 h until day 21. Control rats

received equivalent volume of vehicle (i.p.). SPS and SPS + JTC-801-treated animals were individually housed throughout the 21 day study; all others were housed with a cage mate.

## Elevated plus maze (EPM) test

Rats were tested on the EPM as previously described on day 9 after SPS to determine the effect of JTC-801 treatment on the appearance of anxiety symptoms (Zhang *et al.*, 2012). EPM tests were arranged between 09:00 and 10:30 h before the pain assessment. The plus maze consisted of two open (50 cm × 10 cm) and two closed (50 cm × 10 cm × 40 cm) arms elevated 40 cm above floor with average light levels 40–55 lux. Each rat was placed in the centre of the apparatus facing the closed arms. The exposure was recorded with a video camera for 5 min and analysed by Any-maze software (Stoelting Co., Wood Dale, IL, USA). The total mobile time, travelled distance, entries into closed arms and the time spent in the open arms were calculated.

## Nociceptive sensitivity tests

A plantar analgesia meter (IITC Life Science Inc., Woodland Hills, CA, USA) was utilized to measure paw withdrawal latency (PWL) to an infrared light beam (thermal sensitivity) directed towards each paw with the lamp set at 25% active intensity. Cut-off time was set at 30 s to prevent tissue damage. An Electronic von Frey anesthesiometer (IITC Life Science, Inc.) was utilized for mechanical nociception assessment. Rats were placed in clear plastic boxes with a wire mesh floor and paw withdrawal thresholds (PWT) were obtained from the mid-plantar aspect of each hind paw. The responses to thermal and mechanical stimuli were tested 2 h apart. The average of three assessments spaced 5 min apart were compared between groups for each test. Baseline pain threshold was assessed 1 day before SPS was initiated. After 7 days of isolation, pain sensitivity was assessed between 11:00 and 15:00 h on days 7, 9, 12, 14, 18 and 21. Rats were weighed before each pain test. The experimental paradigm is illustrated by the scheme in Figure 1. Rats were killed with

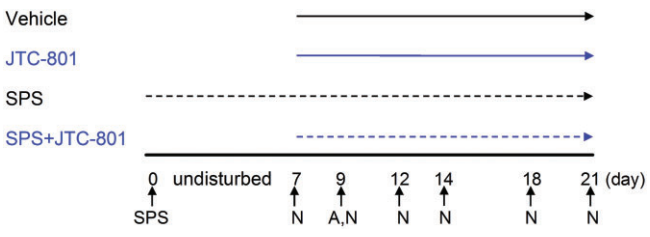



**Figure 1**

Experimental paradigm. Rats were assessed for baseline nociceptive sensitivity (N) to mechanical and thermal stimuli as described above, prior to SPS initiation at day 0. After 7 days of isolation, nociceptive sensitivity was assessed (days 7, 9, 12, 14, 18 and 21). Anxiety-like behaviour (A) was tested by EPM on day 9. SPS rats remained in isolation throughout the paradigm. From day 7 to 21 after SPS, JTC-801 was administered once daily (i.p. 6 mg $kg^{-1}$ in 3% DMSO and 0.05% hydroxypropyl cellulose) to JTC or SPS + JTC groups; vehicle and SPS groups received the same volume of vehicle. Rats were killed at day 21; sera, CSF and brain samples were taken immediately for further analysis.

Beuthanasia (i.p. 0.22 mg $kg^{-1}$, Schering-Plough Animal Health, Union, NJ, USA) on day 21. Serum, CSF and brain samples were immediately collected for subsequent biochemical analysis.

## [$^{35}$S]-GTPγS binding assay

NOP and μ-opioid receptor activities were determined by [$^{35}$S]-GTPγS binding assay. Brain was dissected in ice-cold Krebs buffer and ~300 mg cortical tissue was collected from each of four naïve rats. Membrane protein was prepared as described (Odagaki and Toyoshima, 2006). Brain tissue was homogenized in 5 mL of ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 10% (w/v) sucrose and centrifuged at 1000× *g* for 10 min. The supernatant was washed twice by centrifugation at 9000× *g* for 20 min and re-suspended in 5 mL of TED buffer. The suspension was kept on ice for 30 min, followed by centrifugation at 35 000× *g* for 10 min. Pellet was stored at –80°C until use. [$^{35}$S]-GTPγS binding assay was conducted as described (Zhang *et al.*, 2012). Aliquots (25 μL) of the diluted rat brain membranes equivalent to 10 μg protein were incubated at 25°C for 60 min in 100 μL of 50 μM Tris-HCl buffer containing 0.3 nM [$^{35}$S]-GTPγS, 0.5% BSA, 10 μM GDP, 0.1 μM EDTA, 0.2 μM DTT, 5 μM $MgCl_2$ and 100 μM NaCl in the presence of $10^{-9}$–$10^{-5}$ M N/OFQ or DAMGO. Adenosine deaminase (20 units $L^{-1}$) was added to eliminate endogenous adenosine and reduce basal [$^{35}$S]-GTPγS binding (Moore *et al.*, 2000). The reaction was terminated by rapid filtration through glass fiber filters using a Brandel cell harvester with three washes with 5 mL of ice-cold washing buffer (5 mM $KPO_4$, pH 7.4). Radioactivity was determined by liquid scintillation spectroscopy. Non-specific binding was measured in the presence of 100 μM unlabelled GTPγS, which was subtracted from the total binding to define the specific [$^{35}$S]-GTPγS binding.

## Tissue preparation and radioimmunoassay

Rat brains were sliced (600–800 μm) in ice-cold Krebs buffer using a vibratome (OTS-4000, Electron Microscopy Sciences, Hatfield, PA, USA). PAG, hippocampus, amygdala, hypothalamus, prefrontal cortex and lumbar spinal cord dorsal horn were dissected according to rat brain atlas (Paxinos, 2005). Brain tissues were homogenized in 4°C distilled water and distributed into three aliquots for peptide analysis, cell lysate and mRNA preparation. The procedure for peptide extraction was described previously (Zhang *et al.*, 2012). Briefly, acetic acid (0.5 M, 300 μL) was preheated to 95°C, added to each sample of brain tissue and boiled for 10 min before cooling on ice for 2 min. Samples were homogenized and reheated at 95°C for 5 min, cooled on ice, then centrifuged at 15 000× *g* for 15 min at 4°C. Supernatant was dried in a vacuum centrifuge, and stored at –80°C until assay. Blood was withdrawn from the heart with an 18-gauge needle between 1500 and 1700 h, and maintained at room temperature for 30 min. Blood samples were centrifuged at 5000× *g* at 4°C for 5 min, serum was collected and stored at –80°C. CSF was withdrawn by inserting a 26-gauge needle into the cysterna magna and immediately stored at –80°C. N/OFQ content in sera, CSF and brain tissues was determined by radioimmunoassay (RIA) kit (Phoenix Pharmaceuticals, Belmont, CA, USA) according to

the protocol suggested by the manufacturer. All samples and standards were assayed in duplicate. The sensitivity of the assay was 10 pg $mL^{-1}$; non-specific binding was 2.9%. There was no cross-reactivity with dynorphin A (1–17), enkephalin or β-endorphin. Concentration of soluble protein present in the brain tissue extract was determined by a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Total amount of N/OFQ in brain tissues was calculated and expressed as pg·$mg^{-1}$ protein ± SEM. Total amount of N/OFQ in sera and CSF was expressed as pg $mL^{-1}$. Corticosterone levels in serum were determined by RIA kit (MP Biomedicals, Orangeburg, NY, USA) according to the manufacturer's manual. The sensitivity of the assay was 25 ng $mL^{-1}$ and non-specific binding was 2.6%. Total amount of serum corticosterone was calculated and expressed as ng $mL^{-1}$ (mean ± SEM).

### *Immunoblotting*

Aliquots of homogenized brain tissues for immunoblotting were diluted 1:1 with 2 × RIPA buffer (2% NP40, 1% $Na_2$deoxycholate, 0.2% SDS, 10 mM EDTA, 20 mM NaF, PBS) containing freshly added protease and phosphatase inhibitors and incubated for 30 min on ice, with subsequent centrifugation at 14 000× *g* for 10 min. After protein concentration determination with Pierce BCA protein assay kit, homogenates were solubilized with 4× Laemmli buffer and stored at –80°C. Samples were resolved using SDS-PAGE on 8–15% Tris-glycine gels (~40 µg total protein per well), transferred to polyvinylidene fluoride membranes, blocked with 5% milk in TBS-Tween buffer, and incubated overnight at 4°C with goat anti-KOR3 (A-18) antibody (1:500; sc-9760, Lot #E2507, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibody conjugated to horseradish peroxidase was incubated for 1 h at room temperature in 5% milk in TBS-T. Immunoreactive bands were visualized by chemiluminescence, captured with the Ultralum Omega Imaging System and densitometry was performed using Ultra Quant 6.0. Membranes were rinsed, stripped and re-probed with anti-actin (1:2000; Sigma-Aldrich, St Louis, MO, USA) as an internal loading control.

### *Real-time PCR*

TRI reagent (Sigma, St Louis, MO, USA) was immediately added to aliquots of homogenized brain tissues for PCR at 10 times of volume for mRNA extraction. RNA quality and concentration was determined by Experion RNA StdSens Analysis Kit (Bio-Rad, Hercules, CA, USA). Samples with RNA Quality Index greater than 7 were further examined. cDNA was synthesized to a final concentration of 1 µg $µL^{-1}$ using SuperScript III Reverse Transcriptase (Sigma). Real-time PCR was performed using SYBR Green Master Mix (AnaSpec, Fremont, CA, USA) and 125 nM forward and reverse primers (rat NOP Fwd: 5′-GTT CAA GGA CTG GGT GTT CAG CCA GGT AGT-3′; rat NOP Rev: 5′-TGC TGG CCG TGG TAC TGT CTC AGA ACT CTT-3′; rat GAPDH Fwd: 5′- TGC ACC ACC AAC TGC TTA GC-3′; rat GAPDH Rev: 5′-GGC ATG GAC TGT GGT CAT GAG-3′) and performed in ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The GAPDH gene was used as an internal normalizer. Data was analysed using the comparative Ct method, and compared with control values (Schmittgen and Livak, 2008).

### *Data analysis*

RIA curves and data were analysed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA). Data are expressed as means ± SEM. Statistical comparisons of behavioural and neurochemical data were performed by one-way or two-way ANOVA followed by Bonferroni's *post hoc* analysis using GraphPad Prism 6.0 software. Results were considered statistically significant if $P < 0.05$.

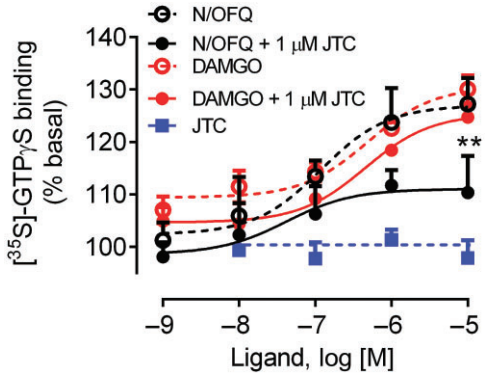
### *Materials*

JTC-801 was supplied by Tocris Bioscience, Bristol, UK; DAMGO and N/OFQ by the NIDA Drug Supply Program and adenosine deaminase by Sigma Aldrich.

## Results

### *JTC-801 attenuated N/OFQ-stimulated [$^{35}$S]-GTPγS binding in rat brain*

JTC-801 is a selective small-molecule NOP receptor antagonist showing good bioavailability and blood–brain barrier permeability (Shinkai *et al.*, 2000). Its NOP receptor binding specificity and antagonism of N/OFQ-suppression of forskolin-stimulated cAMP accumulation was demonstrated in membranes from cell lines expressing recombinant NOP receptors (Shinkai *et al.*, 2000; Yamada *et al.*, 2002). While JTC-801 binding selectivity in rat cerebrocortical membranes has been reported (Yamada *et al.*, 2002), JTC-801 inhibition of direct N/OFQ-stimulated NOP receptor GTPγS binding activity in rat brain membranes has not. Therefore, we tested the ability of JTC-801 to inhibit N/OFQ- and DAMGO-mediated increases in [$^{35}$S]-GTPγS binding in rat brain membranes. As illustrated in Figure 2, N/OFQ dose dependently stimulated




## Figure 2

Effect of JTC-801 on N/OFQ and DAMGO-stimulated [$^{35}$S]-GTPγS binding in rat brain. Brain membranes (10 µg) from naïve rats ($n = 4$) were incubated with and without agonist and JTC-801 to determine the ability of JTC-801 to block N/OFQ- and DAMGO-mediated increases in [$^{35}$S]-GTPγS binding, and to alter basal binding. Both N/OFQ and DAMGO ($10^{-9}$–$10^{-5}$ M) concentration-dependently stimulated [$^{35}$S]-GTPγS binding in brain membranes. JTC-801 (1 µM) reversed the stimulatory effect of N/OFQ without altering N/OFQ potency. JTC-801 failed to reverse the stimulatory effect of DAMGO or to alter basal [$^{35}$S]-GTPγS binding. **$P < 0.0015$.

[$^{35}$S]-GTPγS binding in rat cortical membranes with $E_{max}$ = 127 ± 2.2% and $EC_{50}$ = 118 nM. When combined with JTC-801 (1 μM), N/OFQ-stimulated [$^{35}$S]-GTPγS binding was attenuated 59% ($E_{max}$ = 111 ± 1.9%) without altering N/OFQ potency, as determined by Student's *t*-test ($P$ = 0.0015, $n$ = 4). DAMGO dose dependently stimulated [$^{35}$S]-GTPγS binding in brain membranes ($E_{max}$ = 131 ± 2%; $EC_{50}$ = 486 nM); inclusion of JTC-801 (1 μM) had no effect on DAMGO parameters ($P$ = 0.1119, $n$ = 3; $E_{max}$ = 125 ± 2%; $EC_{50}$ = 450 nM). JTC-801 alone had no effect on [$^{35}$S]-GTPγS binding, consistent with an absence of partial agonist properties at NOP and other opioid receptors (Sestili *et al.*, 2004). This also suggests a lack of partial agonist effect on any other GPCR present in cortical membranes.

## *JTC-801 reversed allodynia and hyperalgesia after SPS*

We first reported persistent mechanical allodynia and thermal hyperalgesia after SPS that was accompanied by anxiety-like symptoms, hypocortisolism and increased N/OFQ in CSF and serum (Zhang *et al.*, 2012). To determine if increased N/OFQ contributed to the maintenance of SPS-induced nociceptive hypersensitivity/allodynia, PWT for mechanical nociceptive stimuli and PWL to thermal nociceptive stimuli were measured in vehicle or SPS rats treated with or without JTC-801 from days 7–21 as illustrated in Figure 3. There was no difference in PWT between rats receiving vehicle or JTC-801 injection over the 21 day study, indicating that i.p. JTC-801 had no effect on mechanical sensitivity in naïve rats. *Post hoc* tests did not reveal difference in sensitivity within the Veh-treated group over the 21 day period, indicating that the rats were not sensitized to repeated assessments. Two-way ANOVA revealed a significant interaction between SPS and time on PWT [$F$(6, 108) = 18.15, $P$ < 0.0001] with Veh and SPS rats, indicating that changes in PWT differed in the two groups over time. Basal pain thresholds to electronic von Frey stimuli were equivalent between groups. However, as early as day 7, SPS-exposed rats exhibited a decreased PWT in both hind paws compared with their pre-SPS thresholds and to PWT in Veh rats (only left hind paw data are shown). Allodynia was sustained in SPS rats throughout the remainder of the 21 day study as revealed by *post hoc* analysis. When treated daily with JTC-801, PWT in SPS rats gradually increased over time; two-way ANOVA revealed significant interaction between JTC-801 and time [$F$(6, 127) = 11.89, $P$ < 0.0001], and a significant effect of JTC-801 [$F$(1, 127) = 175.9, $P$ < 0.0001] and time [$F$(6, 127) = 51.53, $P$ < 0.0001] individually on PWT (Figure 3A). *Post hoc* analysis indicated that JTC-801-treated SPS rats exhibited significantly higher PWT by day 9, and allodynia was totally blocked by day 21. This suggests that N/OFQ contributed to maintenance of mechanical allodynia induced by SPS.

Similar results were noted with thermal stimuli (Figure 3B). No difference in PWL between Veh and JTC-801-treated groups was detected. Significant interaction between SPS and time [$F$(6, 108) = 10.18, $P$ < 0.0001] indicated that changes in PWL differed over time between Veh and SPS rats receiving vehicle injection. Two-way ANOVA revealed a significant effect of SPS [$F$(1, 108) = 142.2, $P$ < 0.0001] and time [$F$(6, 108) = 6.191, $P$ < 0.0001] on PWL to radiant heat. Decreased PWL of the left hind paw to thermal stimuli emerged at day 7 in SPS rats and lasted through day 21 compared with pre-SPS threshold and to Veh rats. Two-way ANOVA revealed significant interaction between JTC-801 and time [$F$(6, 127) = 10.68, $P$ < 0.0001], as well as significant effects of JTC-801 [$F$(1, 127) = 67.44, $P$ < 0.0001] and of time [$F$(6, 127) = 11.49, $P$ < 0.0001] on PWL. PWT in JTC-801-treated SPS rats returned to control level at day 14, consistent with a contribution of N/OFQ to maintenance of thermal hyperalgesia in SPS.

## *JTC-801 reversed SPS-induced anxiety-like behaviour*

Our previous study confirmed anxiety-like behaviour in the SPS model at day 9 (Zhang *et al.*, 2012). To examine the role of N/OFQ on the appearance of anxiety symptoms, rats were subjected to EPM test at day 9 after SPS +/– JTC-801 treatment (Figure 4). Two-way ANOVA with Bonferroni's *post hoc* analysis

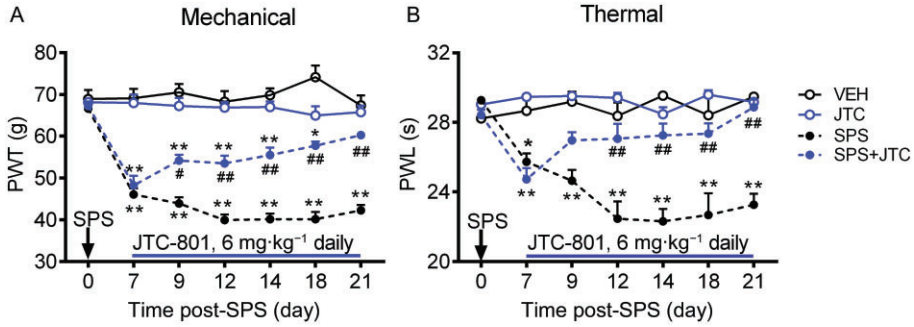
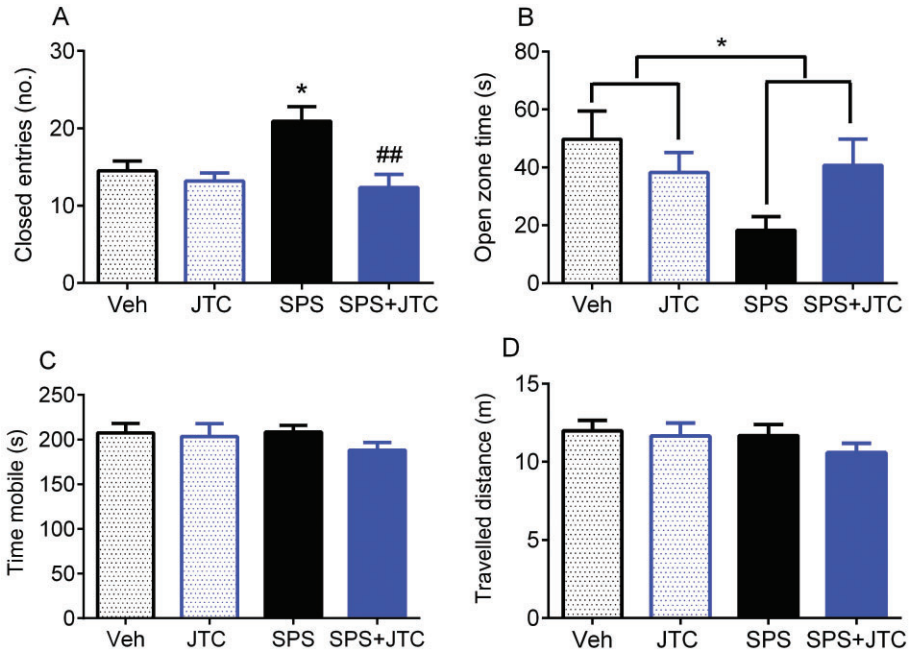



**Figure 3**

JTC-801 reversed SPS-induced mechanical allodynia and thermal hyperalgesia. Rats were randomly divided into four groups: vehicle ($n$ = 9), JTC-801 ($n$ = 10), SPS ($n$ = 12) and SPS + JTC-801 ($n$ = 12). SPS procedures were performed at day 0. Beginning at day 7 after SPS, rats were injected with JTC-801 (6 mg kg$^{-1}$ per day i.p.) until day 21, when rats were killed. Mechanical (A) and thermal (B) nociceptive sensitivity in both hind paws was assessed on days 0, 7, 9, 12, 14, 18 and 21, and both exhibited similar responsiveness. For clarity, only results from the left hind paw are shown. Data are plotted as means ± SEM. *$P$ < 0.05, **$P$ < 0.01 vs. VEH; #$P$ < 0.05, ##$P$ < 0.01, SPS vs. SPS + JTC; two-way ANOVA followed by Bonferroni's *post hoc* test.




## Figure 4

Effect of JTC-801 on SPS-induced anxiety-like behaviours. Rats were randomly divided into four groups: vehicle ($n$ = 9), JTC-801 ($n$ = 10), SPS ($n$ = 12) and SPS + JTC-801 ($n$ = 12) and injected with vehicle or JTC-801 (6 mg kg$^{-1}$ per day, i.p.) from days 7 ~ 21. The appearance of anxiety-like behaviours was assessed on day 9. SPS-exposed rats made fewer entries into closed arms (A) and spent significantly less time in open arms (B). The SPS-induced anxiety-like behaviour was blocked by JTC-801 treatment. JTC-801 alone did not show any effect on any of the observed indices. *$P$ < 0.05, SPS vs. VEH; ##$P$ < 0.01, SPS + JTC vs. SPS; two-way ANOVA followed by Bonferroni's *post hoc* test. No significant difference in total mobile time (C) or travelled distance (D) between control and SPS rats was observed.

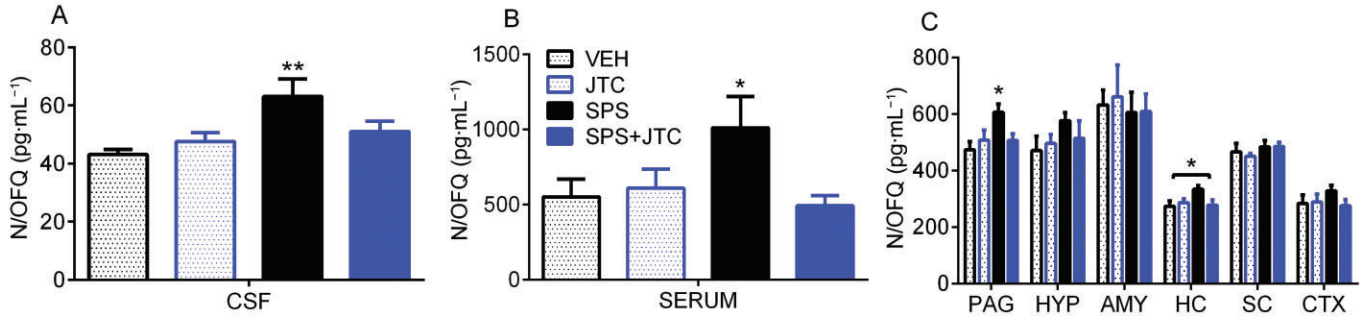
revealed a significant interaction between SPS and JTC-801 [$F$(1, 43) = 5.566, $P$ = 0.023] and a significant effect of JTC-801 on entries into closed arms [$F$(1, 43) = 10.34, $P$ = 0.0025] (Figure 4A). Two-way ANOVA also indicated a significant interaction between SPS and JTC-801 on spent time in the open arms (Figure 4B) as decreased time in open arms by SPS rats was reversed by JTC [$F$(1, 43) = 4.542, $P$ = 0.039]. These anxiety-like behaviours cannot be explained by reduced mobility, as locomotor function was not impaired by SPS; both groups spent equivalent mobile time (Figure 4C) and travelled a comparable distance (Figure 4D). JTC-801 alone did not affect rat behaviour, as there was no difference in any parameter measured between control rats receiving JTC-801 or vehicle treatment.

### *Effect of SPS and JTC-801 on N/OFQ levels*

Our previous report suggested that maintenance of allodynia and hyperalgesia are associated with elevated N/OFQ levels in serum and CSF after SPS (Zhang *et al.*, 2012). In this study, we examined the effect of JTC-801 on SPS-induced changes of N/OFQ levels in serum, CSF and in various brain regions associated with anxiety and/or pain processing. As expected, increased N/OFQ content appeared in CSF of SPS rats at day 21 (Figure 5A), compared with control. This increase was reversed after JTC-801 treatment. Two-way ANOVA revealed significant interaction of SPS and JTC-801 [$F$(1, 28) = 4.453, $P$ = 0.044] and significant effect of SPS [$F$(1, 28) = 8.804, $P$ = 0.006] on N/OFQ levels in CSF (Figure 5A). A similar change was observed in serum (Figure 5B), where elevated N/OFQ levels appeared in SPS rats at day 21, compared with control. This increase was reversed by JTC-801. Two-way ANOVA revealed significant interaction of SPS and JTC-801 [$F$(1, 29) = 4.302, $P$ = 0.047] on N/OFQ levels in serum (Figure 5B). In brain tissues, elevated N/OFQ levels were observed in PAG and hippocampus of SPS rats at day 21: two-way ANOVA revealed significant interaction of SPS and JTC-801 on N/OFQ levels in PAG [$F$(1, 29) = 4.895, $P$ = 0.035] and hippocampus [$F$(1, 29) = 4.521, $P$ = 0.042], and a significant effect of SPS in PAG [$F$(1, 29) = 4.675, $P$ = 0.039]. SPS did not increase N/OFQ levels in hypothalamus, amygdala, spinal cord and prefrontal cortex at this time point (Figure 5C). JTC-801 alone failed to alter N/OFQ levels in serum, CSF or any brain region tested.

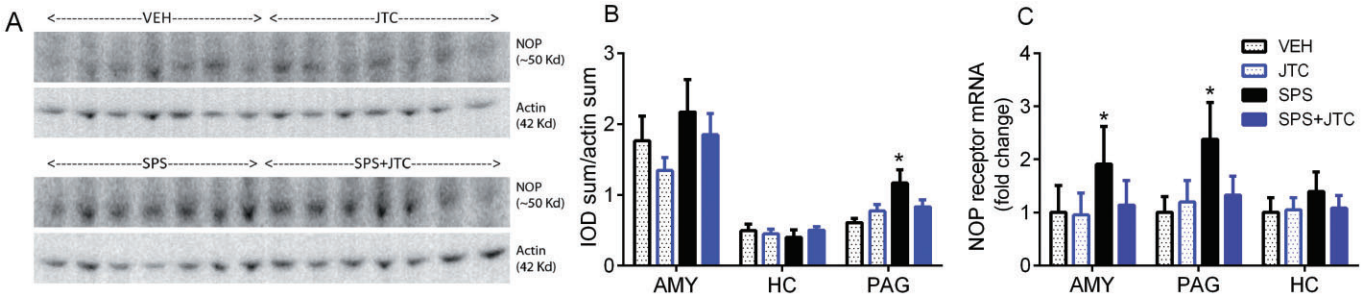
### *Effect of SPS and JTC-801 on NOP receptor expression*

To determine the effect of SPS and JTC-801 on NOP receptor expression, cell lysates from amygdala, hippocampus and PAG were prepared for immunoblotting (Figure 6A). Two-way ANOVA of immunoblot densitometric analysis revealed significant interaction between SPS and JTC-801 treatment [$F$(1, 24) = 4.367, $P$ = 0.047] and a significant effect of SPS [$F$(1,24) = 6.585, $P$ = 0.017] on NOP receptor expression in PAG (Figure 6B). No change was noted in amygdala or hippocampus. To determine if protein changes were a reflection of increased NOP receptor message, mRNA from amygdala, hippocampus and PAG were subjected to real-time PCR analysis.




## Figure 5

Effect of JTC-801 on CSF, serum and brain N/OFQ levels in SPS rats. N/OFQ content in CSF (A), serum (B) and brain regions (C) from day 21 was determined by RIA ($n$ = 8 in vehicle, SPS and SPS + JTC-801 groups; $n$ = 9 in JTC-801 group). Data are plotted as mean ± SEM. *$P$ < 0.05, **$P$ < 0.01Two-way ANOVA followed by Bonferroni's *post hoc* test showed significant interaction between SPS and JTC-801 in serum, CSF, PAG and hippocampus, and a significant effect of SPS on N/OFQ levels in CSF and PAG. No differences in N/OFQ content was noted between groups in hypothalamus (HYP), amygdala (AMY), spinal cord (SC) and prefrontal cortex (CTX) at day 21.




## Figure 6

Effect of SPS and JTC-801 on NOP receptor gene expression. mRNA and cell lysates prepared from amygdala (AMY), hippocampus (HC) and PAG were subjected to immunoblotting and real-time PCR. (A) Representative immunoblots of NOP receptor and actin immunoreactivity in PAG from all four groups ($n$ = 7/group). (B) Immunoblots were quantified by densitometric analysis and normalized to β-actin Data are presented as mean ± SEM. *$P$ < 0.05; two-way ANOVA followed by Tukey's *post hoc* test showed a significant effect of SPS on NOP receptor expression in PAG. (C) Fold changes in NOP receptor mRNA in SPS, JTC-801 and SPS + JTC treated rats were normalized to vehicle-treated controls as described in Methods. The GAPDH gene was used as an internal control. SPS increased NOP receptor mRNA expression in AMY and PAG. Data are presented as means ± SEM. *$P$ < 0.05; two-way ANOVA analysis ($n$ = 8 in vehicle, SPS and SPS + JTC-801 groups; $n$ = 9 in JTC-801 group).

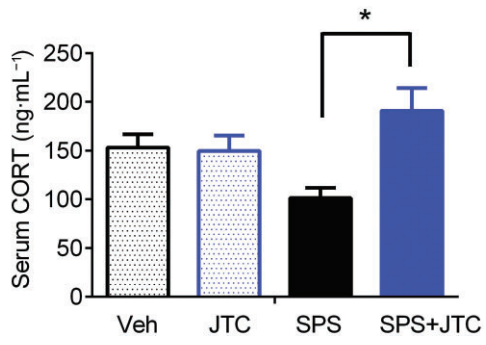
Two-way ANOVA revealed significant effect of SPS on NOP receptor mRNA levels in amygdala [$F(1, 28) = 4.689$, $P = 0.039$] and PAG [$F(1, 28) = 5.169$, $P = 0.03$], indicating that SPS up-regulated NOP receptor mRNA expression in these two brain regions (Figure 6C). No change was noted in hippocampal NOP receptor mRNA. JTC-801 alone did not alter NOP receptor expression in any of the brain regions tested.

### *JTC-801 reversed SPS-induced hypocortisolism*

We previously reported that serum corticosterone levels remained unchanged at days 9 and 14 of SPS, but decreased by day 28 (Zhang *et al.*, 2012). In the current study, the effect of JTC-801 on SPS-induced decreases in serum corticosterone was determined at day 21 (Figure 7). Mean serum corticosterone levels were comparable in control rats receiving vehicle or JTC-801 injection, suggesting that JTC-801 had no effect on serum corticosterone level in non-SPS rats. Similar to what we reported for day 28 of SPS, SPS-treated rats exhibited 33% lower corticosterone levels in serum on day 21, compared with control rats receiving vehicle injection. This low corticosterone level was increased 25% by JTC-801 treatment, compared with vehicle-treated controls. Two-way ANOVA revealed a significant interaction between SPS and JTC-801 [$F(1,30) = 8.177$, $P = 0.0076$] and a significant effect of JTC-801 [$F(1,30) = 6.926$, $P = 0.0133$], consistent with a role of N/OFQ in SPS-induced hypocortisolism.

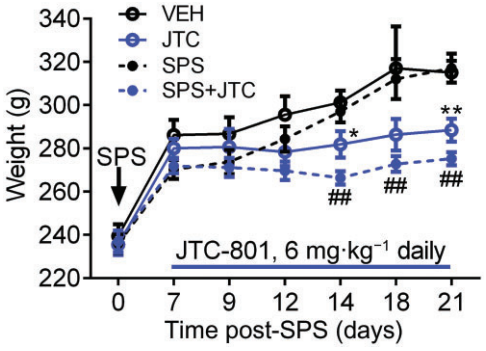
### *JTC-801 blocked body weight gain in rats*

Body weight was monitored before each pain assessment. All rats were randomly divided into one of four groups, with equal body weight in each group prior to initiation of SPS. SPS-treated rats appeared to have lower body weight than control rats after 7 days isolation, although no statistical difference were detected. When injected with vehicle, SPS rats gained body weight and reached same weight by day 21 as rats in the vehicle group, confirming that body weight in the




## Figure 7

Effect of SPS and JTC-801 treatment on serum corticosterone (CORT) levels. Corticosterone levels in serum samples were determined by RIA at day 21 from Veh and SPS rats treated with or without JTC-801 (i.p., 6 mg $kg^{-1}$ per day from days 7–21; $n = 8$ in vehicle, SPS and SPS + JTC-801 groups, $n = 9$ in JTC-801 group). JTC-801 alone had no effect on serum corticosterone levels, but corticosterone levels in SPS + JTC rats were reversed to a level higher than in vehicle-treated rats. Data are plotted as means ± SEM. *$P < 0.05$; two-way ANOVA followed by Bonferroni's *post hoc* test.




## Figure 8

JTC-801 prevents weight gain in both vehicle and SPS-treated rats. Rats were randomly divided into four groups as described above: vehicle ($n = 9$), JTC-801 ($n = 10$), SPS ($n = 12$) and SPS + JTC-801 ($n = 12$). SPS procedures were performed at day 0. Beginning at day 7 after SPS, rats were injected with JTC-801 (6 mg $kg^{-1}$ per day i.p.) until day 21. Rats were weighed prior to each nociception testing. Data are plotted as means ± SEM. *$P < 0.05$, **$P < 0.01$, JTC vs. VEH; ##$P < 0.01$, SPS + JTC vs. SPS; two-way ANOVA followed by Bonferroni's *post hoc* test.

long term was not affected in this PTSD model. However, weight gain slowed in all JTC-801-treated rats (Figure 8). After 7 days of JTC-801 injection, both control and SPS rats had significantly lower body weight than their counterparts receiving vehicle injection. Two-way ANOVA revealed significant interaction between JTC-801 and time [$F(18, 240) = 2.545$, $P = 0.007$] and significant effect of JTC-801 [$F(6, 240) = 54.33$, $P < 0.0001$] and of time [$F(3, 240) = 22.03$, $P < 0.0001$] on body weight, indicating that body weight gain was blocked by JTC-801 treatment. Weight differences did not seem to alter the animal's general health, mobility or activity as observed throughout the experimental period.

# Discussion

Our current findings suggest that a dysregulation of the N/OFQ-NOP receptor system contributes to anxiety-like behaviours and hypocortisolism, and maintenance of SPS-induced allodynia and hyperalgesia. We previously reported that N/OFQ levels in the CSF increased at day 9, subsequent to the appearance of allodynia and hyperalgesia (Zhang *et al.*, 2012). Here, we have examined levels of N/OFQ and the NOP receptor in brain regions associated with these symptoms: PAG and spinal cord (allodynia), hippocampus and amygdala (anxiety), amygdala and hypothalamus (hypocortisolism) and frontal cortex. At day 21 in SPS rats, N/OFQ levels were elevated in CSF and serum, as expected based upon previous findings, and they were elevated in PAG and hippocampus as well.

Endogenous N/OFQ levels in serum and/or CSF are increased in humans and *in vivo* models of acute and chronic pain and stress (Ko *et al.*, 2002; Cornefjord *et al.*, 2004; Joseph *et al.*, 2007; Liu *et al.*, 2012). Several studies have examined the effect of acute or mild chronic stress on N/OFQ expression in CSF, serum and/or brain, but few reports examined all parameters with a more severe, chronic stress. Acute restraint stress accelerated endogenous N/OFQ release and biosynthesis in basal forebrain (Devine *et al.*, 2003), and enhanced N/OFQ expression in hippocampus (Nativio *et al.*, 2012), while repeated restraint stress increased ppN/OFQ mRNA in bed nucleus and thalamus and decreased it in amygdala (Delaney *et al.*, 2012). Regardless of the differences between studies, all support the possibility that dysfunction of the N/OFQ system may contribute to behavioural and hormonal dysregulation following stress.

Though it may seem counter-intuitive that the NOP receptor was not down-regulated by increased levels of N/OFQ, the SPS paradigm produces a complex array of changes (Yamamoto *et al.*, 2009), any of which may directly or indirectly modulate NOP receptor expression. Though regulation of NOP receptor gene expression is not well studied, what is known fits with our findings. First, NOP receptor expression increases in persistent pain models (Briscini *et al.*, 2002; Ma *et al.*, 2005). The endogenous N/OFQ-NOP receptor system appears to become tonically active at the level of the ventrolateral PAG during chronic pain (Scoto *et al.*, 2009), which suggests that tonic activity is maintained by turning on NOP receptor gene expression. Second and equally important is that NOP receptor gene expression can be modulated by stress. Social defeat stress elevated NOP receptor mRNA in amygdala of rats (Green and Devine, 2009), while immobilization stress down-regulated NOP receptor mRNA in amygdala of a mouse model of dysregulated fear (Andero *et al.*, 2013). The SPS model includes three additional stressors in addition to restraint/immobilization. Unlike the previous studies, we measured functional NOP receptor expression as well as its mRNA. The current study confirms that blockade of NOP receptor activity reverses SPS-induced symptoms, but future studies will examine the mechanism underlying NOP receptor increases.

JTC-801 is a relatively selective, low MW NOP receptor antagonist showing good bioavailability and blood–brain barrier permeability (Shinkai *et al.*, 2000). Its NOP receptor binding specificity and antagonism of N/OFQ-suppression of

forskolin-stimulated cAMP accumulation was demonstrated in membranes from cell lines expressing recombinant human NOP, μ-, κ- and δ-opioid receptors, in which it displayed about 12.5-, >120- and >975-fold binding selectivity for NOP receptors over μ-, κ- and δ-receptors respectively (Shinkai *et al.*, 2000). In rat cerebrocortical membranes, JTC-801 also displayed higher binding affinity for the NOP receptor ($IC_{50}$ = 472 nM) than for the μ-opioid receptor ($IC_{50}$ = 1831 nM; Yamada *et al.*, 2002). Furthermore, the antinociceptive effect of JTC-801 was not blocked by naloxone, indicating that JTC-801 did not produce its antinociceptive actions through μ-opioid receptors (Shinkai *et al.*, 2000; Yamada *et al.*, 2002). Consistent with those studies, our functional NOP and μ-opioid receptor assays indicated that 1 μM JTC-801 inhibited only N/OFQ-, but not DAMGO-, mediated increases in [$^{35}$S]-GTPγS binding in rat brain membranes. JTC-801 exhibited no stimulation of [$^{35}$S]-GTPγS binding alone, indicating that it has no agonist or partial agonist actions in rat cortical membranes, further ruling out a partial agonist effect at the μ-opioid receptor. Indeed, if endogenous μ-opioid agonists were released during the SPS paradigm and JTC-801 antagonized their actions, one would expect to see enhanced hyperalgesia and allodynia with JTC treatment – and that was not the case. Even if JTC-801 bound to the μ-opioid receptor, its low affinity suggests that it would rapidly dissociate and not produce antinociception 16–18 h after each administration. However, results from binding and *in vivo* experiments suggest that JTC-801 has competitive and non-competitive binding properties at the NOP receptor that may account for its long-lasting antinociceptive effects (Yamada *et al.*, 2002; Mabuchi *et al.*, 2003; Marti *et al.*, 2003; Sestili *et al.*, 2004; Del Giudice *et al.*, 2011).

We first reported SPS-induced long-lasting hyperalgesia and allodynia was accompanied by elevated N/OFQ levels in serum (day 28) and CSF (days 9, 14 and 28), suggesting a link between N/OFQ and the maintenance of hyperalgesia and allodynia in a rat model of PTSD (Zhang *et al.*, 2012). Concurrent with sustained mechanical allodynia and thermal hyperalgesia, our current results showed that N/OFQ content in serum and CSF also was increased at day 21 after SPS (Figure 5A and B), consistent with our previous findings. Increased N/OFQ may originate from multiple cell types and brain regions (Lambert, 2008); we examined N/OFQ content and NOP receptor expression in brain regions important in pain, anxiety and stress modulation. N/OFQ levels were significantly elevated in hippocampus and PAG at day 21 in SPS rats, but not in hypothalamus, amygdala, prefrontal cortex and spinal cord (Figure 5C). SPS up-regulated NOP receptor mRNA levels in PAG and amygdala, and increased NOP receptor protein expression in PAG (Figure 6). N/OFQ and NOP receptor up-regulation was reversed by JTC-801 treatment at the time at which allodynia and hyperalgesia had abated, but JTC-801 alone had no effect on nociceptive sensitivity or N/OFQ or NOP receptor levels in naïve rats (Figures 3, 5 and 6). The PAG is one of the most important brain regions in regard to nociceptive sensitivity, controlling the descending analgesic pathway. Increased NOP receptor levels in the PAG, in particular, correlate very closely with hyperalgesia and allodynia associated with activation of supraspinal NOP receptors by N/OFQ (Heinricher *et al.*, 1997; Tian *et al.*, 1997; Pan *et al.*, 2000; Tamai *et al.*, 2005; Joseph *et al.*, 2007). Inhibition of supraspinal N/OFQ-induced hyperalgesia and/or allodynia has been reported with several NOP receptor antagonists other than JTC-801, including J-113397 (Ozaki *et al.*, 2000), [NPhe$^1$]nociceptin (1–13) $NH_2$ (Corradini *et al.*, 2001), SB-612111 (Zaratin *et al.*, 2004) and UFP-101 (Scoto *et al.*, 2009). Enhanced N/OFQ-NOP receptor activity in PAG, hippocampus and amygdala after SPS may maintain hypersensitivity that was initially a result of the prolonged stressor or neuropathy (Cornefjord *et al.*, 2004; Joseph *et al.*, 2007; Khroyan *et al.*, 2011; Liu *et al.*, 2012). When rats started to receive JTC-801 treatment at day 7 of SPS, thermal and mechanical hypersensitivity already were established. However, JTC-801 began to reverse nociceptive hypersensitivity, with gradually increasing PWT and PWL and complete block of mechanical allodynia and thermal hyperalgesia by day 21 and 14, respectively (Figure 3), as expected from previous reports in rats (0.01–0.1 mg kg$^{-1}$ i.v., 1–10 mg kg$^{-1}$ p.o., 3–30 mg kg$^{-1}$: Yamada *et al.*, 2002; Mabuchi *et al.*, 2003; Suyama *et al.*, 2003; Tamai *et al.*, 2005).

As with its ability to modulate pain and weight gain, the effect of N/OFQ on anxiety and stress is bidirectional. N/OFQ (i.c.v.) or NOP receptor agonists produce anxiogenic- (Devine *et al.*, 2001; Fernandez *et al.*, 2004; Vitale *et al.*, 2006; Green *et al.*, 2007), as well as anxiolytic-like effects (Jenck *et al.*, 2000; Gavioli *et al.*, 2007; Uchiyama *et al.*, 2008; Lu *et al.*, 2011; Goeldner *et al.*, 2012). These differences may result, in part, from different baseline stress levels between studies, species or strain differences and/or site and amount of N/OFQ or NOP agonist receptor administration. The NOP receptor antagonist, UFP-101, also produced anxiolytic effects in rats (Duzzioni *et al.*, 2011). Because SPS-exposed rats displayed symptoms of anxiety and elevated levels of N/OFQ in CSF on day 9 (Zhang *et al.*, 2012), we hypothesized that treatment with a NOP receptor antagonist would reduce symptoms of anxiety if they resulted from elevated N/OFQ. Indeed, SPS-induced anxiety-like behaviour was blocked by JTC-801 treatment (Figure 4). Our results provide new evidence that daily systemic administration of JTC-801 effectively reversed pain hypersensitivity and anxiety symptoms induced by SPS, without altering baseline nociceptive sensitivity or mobility. It suggests that N/OFQ plays an important role in maintenance of exaggerated nociceptive sensitivity and anxiety in this animal model of PTSD.

PTSD is characterized, in part, by HPA axis dysregulation. N/OFQ activates the HPA axis following acute administration or mild stress as evident by increased circulating adrenocorticotropic hormone and corticosterone (Fernandez *et al.*, 2004; Leggett *et al.*, 2006; Vitale *et al.*, 2006; Green *et al.*, 2007); HPA axis activation by N/OFQ is blocked by NOP receptor antagonist UFP-101 (Leggett *et al.*, 2006; Vitale *et al.*, 2006). As noted above, the role of N/OFQ in HPA axis function after prolonged stress (>4–6 h) is not well established. Our previous data demonstrated hypocortisolism at day 28, but not day 9 and 14, after initiation of SPS, which is consistent with many clinical findings (Meewisse *et al.*, 2007; Morris *et al.*, 2012). Serum corticosterone levels negatively correlated with serum N/OFQ at day 28, suggesting an inverse relationship between N/OFQ and chronic stress (Zhang *et al.*, 2012). In the current study, we demonstrated that serum corticosterone levels were significantly reduced by day 21 after SPS, and hypocortisolism was reversed by JTC-801 treatment (Figure 7). Delaney *et al.*

(2012) previously reported that JTC-801 increased plasma corticosterone in quiescent rats, but the rats in that study were subjected to only a single type of stress (restraint) and not to four diverse forms of stress as utilized in this study. The reversal of serum corticosterone reduction by JTC-801 implies that elevated levels of endogenous N/OFQ noted in the SPS model of PTSD may persistently activate the HPA axis and contribute to its dysregulation.

Reports of body weight changes in animal models of PTSD do not reveal a consistent pattern: weight gain (Cohen *et al.*, 2009; Hammamieh *et al.*, 2012), weight loss (Pulliam *et al.*, 2010) or delayed weight gain (Jia *et al.*, 2012). While rats exposed to SPS seemed to display lower body weight after 7 days of isolation, body weights in SPS rats were not significantly different from Veh-treated control rats at any point in the study. However, manipulation of the N/OFQ-NOP system has been shown to alter food intake. N/OFQ increased food consumption in satiated and food deprived rats, while the NOP receptor antagonist [Nphe$^1$]nociceptin (1–13) $NH_2$ diminished both N/OFQ and deprivation-induced feeding (Olszewski *et al.*, 2010). A previous study utilizing prolonged oral administration of JTC-801 (0.03% and 0.06% w/w in food; Suyama *et al.*, 2003) to rats with chronic constriction injury found no difference in rat body weight between groups, but the effect of JTC-801 on weight of healthy rats was not assessed. The difference between our results and those of Suyama may be attributed to differences in dose, route of JTC-801 administration, the experimental model or some combination of all three. Most recently, Witkin *et al.* (2014) reported a reduction in both feeding and weight gain after daily treatment with the NOP receptor antagonist SB612111 (10 and 30 mg kg$^{-1}$, oral), therefore, it was not too surprising to find that JTC-801 treatment reduced weight gain in rats from both SPS and control groups (Figure 8). There is no evidence that lack of weight gain after JTC-801 administration was related to changes in any of the other variables measured in this study.

Overall, our novel findings demonstrate that JTC-801 reversed SPS-induced nociceptive hypersensitivity and allodynia, anxiety-like behaviour, hypocortisolism and N/OFQ-NOP system up-regulation. The results suggest that elevated supraspinal N/OFQ and NOP receptors play an important role in the development of anxiety and maintenance of hyperalgesia and hypocortisolism in SPS, and that NOP receptor antagonists may be a promising therapeutic approach for treatment of co-morbid PTSD and pain.

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## Author contributions

The experiments were designed and conceived by Y. Z. and K. M. S.; Y. Z. and C. D. S.-D. conducted the experiments; Y. Z., C. D. S.-D. and K. M. S. performed the statistical analyses. Y. Z. and K. M. S. wrote or contributed to the writing of the manuscript. All authors read and approved the final manuscript.

## Conflict of interest

None.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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**Figure S1** JTC-801 does not alter DAMGO-stimulated [$^{35}$S]-GTPγS binding in human SH-SY5Y cells. Membrane protein (10 μg) from SH-SY5Y cells was assayed for the ability of JTC-801 to modulate basal and DAMGO-mediated increases in [$^{35}$S]-GTPγS binding. DAMGO dose dependently increased [$^{35}$S]-GTPγS binding, but the increase was not altered in the presence of 1 μM JTC-801. JTC-801 did not reverse the stimulatory effect of 1 μM DAMGO ($n$ = 2), and did not alter baseline [$^{35}$S]-GTPγS binding when assayed in the absence of DAMGO ($n$ = 3).

**Figure S2** Negative control for NOP receptor antibody. Equal amount of cell lysates from PAG in naïve rats ($n$ = 4) were resolved using SDS-PAGE and transferred to two PVDF membranes. Goat anti-KOR3 (A-18) antibody (1:500) was pre-incubated with its blocking peptide (sc-9760p, Santa Cruz Biotechnology) at ratio of 1:5 at room temperature for 30 min. After blocking with 5% milk, one membrane was incubated overnight at 4°C with goat anti-KOR3 antibody (A: 1:500) while the other membrane was incubated with the antibody that had been pre-incubated with the blocking peptide (B). Secondary antibody conjugated to HRP was incubated for 1 h at room temperature. Membranes were stripped and re-probed with anti-actin (1:2000) as an internal loading control.