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Daniel Artur Abreu Martins

GABA-Dependent Pain Facilitation of Spinal 5-HT3R In Diabetic Neuropathic Pain

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GABA-Dependent Pain Facilitation of Spinal 5-HT3R in Diabetic Neuropathic Pain

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pelo carinho, companheirismo e gargalhadas.

GABA-Dependent Pain Facilitation of Spinal 5-HT3R In Diabetic Neuropathic Pain

Running title: 5-HT3R pronociception in diabetic neuropathic pain

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The authors declare no conflict of interests

What's already known about this topic?

- Activation of 5-HT3 receptors expressed in GABAergic neurons of the spinal cord increases GABA release
- Inhibition of spinal 5-HT3R decreases pain hypersensitivity in animal models of chronic inflammatory and traumatic neuropathic pain

What does this study add?

- Spinal 5-HT3 receptors facilitate pain transmission and contributes to ERKs-mediated spinal sensitization during diabetic neuropathy
- 5-HT3R-mediated pain facilitation during DNP is dependent on spinal GABAergic post-synaptic neurotransmission

Abstract

Background: Spinal 5-HT3 receptors (5-HT3R) has been implicated in chronic pain development. The extent to which 5-HT3R contributes to spinal sensitization and pain during diabetic neuropathy (DN) remain elusive and the mechanisms subserving the effects of 5-HT3R activation on spinal pain processing are still unclear. This study aimed to evaluate the contribution of spinal 5-HT3R to pain facilitation and spinal sensitization during DN. Moreover, considering the pain facilitation mediated by spinal GABA in DN and the increased release of GABA upon 5-HT3R activation, the role of GABA as a mediator of 5-HT3R spinal effect was assessed.

Methods: Mechanical nociception was evaluated by paw pressure test in streptozotocin (STZ)-diabetic and control rats after intrathecal (i.t.) administration of 5-HT3R antagonist (Y25130). The spinal activation of extracellular signal-regulated kinases (ERKs) pathway and the expression of 5-HT3R, glial fibrillary acidic protein (GFAP; marker of astroglia activation) and ionized calcium binding adaptor molecule 1 (IBA-1; marker of microglia activation) were evaluated at the peak maximum effect of Y25130. The involvement of GABA in the behavioural pain effect of Y25130, was assessed in STZ-diabetic animals receiving i.t administrations of muscimol (GABAAR agonist).

Results: Intrathecal administration of Y25130 reverted mechanical hyperalgesia and ERK-mediated spinal sensitization in STZ-diabetic rats, while no effects were observed in control animals. The spinal activation of GABAAR by i.t administration of muscimol abolished Y25130-driven antinociception. The expression of IBA-1, GFAP and 5-HT3R was unaltered by treatment.

Conclusion: These findings point for a GABA-dependent pronociceptive role of spinal 5-HT3R in this chronic pain condition.

Key-Words: Diabetic neuropathic pain; 5-HT3R; GABA; Pain facilitation; ERK1/2, spinal sensitization

1. Introduction

Diabetic neuropathic pain (DNP) is a debilitating complication of diabetes characterized by spontaneous pain, mechanical hyperalgesia and tactile allodynia (Galer et al., 2000). DNP have been mostly attributed to damage of peripheral nerves (Chen and Levine, 2001), however several studies have showed that functional impairments in spinal nociceptive processing account for pain during diabetes (Pertovaara et al., 2001; Chen and Pan, 2002; Morgado and Tavares, 2007; Morgado et al., 2010).

Functional studies using the streptozotocin (STZ)-diabetic rat showed that impaired pain responses are accompanied by spontaneous hyperactivity and hyperexcitability of nociceptive spinal circuits (Morgado and Tavares, 2007; Li et al., 2010). These changes have been attributed to increased peripheral input and recruitment of nociceptive ascending pathways (Burchiel et al., 1985; Chen and Pan, 2002), to alterations of spinal nociceptive modulatory mechanisms (Morgado et al., 2008) and, more recently, to impairments in pain modulation from supraspinal areas (Paulson et al., 2007; Morgado et al., 2011b; Silva et al., 2013). Recent studies showed that the behavioral hypersensitivity and spinal neuronal hyperexcitability are accompanied by persistent activation of descending pain circuits (Morgado et al., 2011b; Silva et al., 2013), namely the descending serotoninergic pathways arising from the rostroventromedial medulla (RVM) (Morgado et al., 2011b). The RVM is a key brainstem relay station of the descending pain modulatory circuits, which modulates spinal pain transmission mainly by the release of serotonin. Depending on the receptor subtype activated and the pain condition serotonin can inhibit or enhance spinal nociceptive transmission (Dogrul et al., 2009). The 5-HT3 receptors (5-HT3R), the only 5-HT ionotropic receptor with excitatory functions, are expressed in the spinal dorsal horn neurons and in the central terminals of primary afferents (Kia et al., 1995). Experimental data on 5-HT3R-mediated modulation of spinal nociceptive processing during acute pain seem conflicting, with studies dividing between anti- and pronociceptive roles (Alhaider et al., 1991; Guo et al., 2014). A pain facilitatory role of spinal 5-HT3R has been demonstrated in animal models of chronic inflammatory and traumatic neuropathic pain (Rahman et al., 2009;

Kim et al., 2014) .

The mechanisms contributing to spinal 5-HT3Rs-mediated pain modulation remain elusive. Studies developed in healthy animals, demonstrated that 5-HT3R activation enhances spinal GABA release, which by acting on GABA receptors inhibits nociceptive transmission and accounts to the attenuated behavioral responses observed after 5HT3R agonist intrathecal administration (Alhaider et al., 1991). However, it should be noted that chronic pain, including DNP, is accompanied by a shift in spinal GABA role, which was shown to exert excitatory instead of inhibitory effects when activating the post-synaptic ionotropic $GABA_A$ receptor ($GABA_AR$) (Jolivalt et al., 2008; Morgado et al., 2008). We hypothesized that GABA release induced by 5-HT3R activation may be eliciting pro-nociceptive effects during DNP, contributing to pain facilitation.

We used the STZ-diabetic rat, a rodent model widely used to study DNP (Calcutt, 2004), to: i) evaluate the contribution of spinal 5-HT3R to pain facilitation and spinal sensitization during DNP; ii) evaluate the involvement of GABA on spinal 5-HT3R-mediated effects on pain modulation in DNP.

2. Material and Methods

2.1. Animals

Male Wistar rats (Charles River, France), weighting 250-350g at the beginning of the experiments were used. The animals were housed 1 per cage with corncob bedding, in a room with controlled environmental conditions (temperature, 22 ± 2ºC; humidity, 55 ± 5%) under a 12-h light/dark cycle (lights on from 8 am to 8 pm). Food (diet ref: A04, SAFE) and water were available ad libitum. The experiments were licensed by the Portuguese Food and Veterinary General Directorate (licence number 0420/000/000/2012) and were performed in accordance with the ethical guidelines of the European Community Council Directive 2010/63/EU and of the International Association for the Study of Pain in conscious animals (Zimmermann, 1983).

2.2. Induction of diabetes

Animals were made diabetic by a single intraperitoneal (i.p.) injection of STZ (60 mg/kg body weight; Sigma-Aldrich, Spain). Age-matched controls received equal volume of vehicle solution (citrate buffer 0.1M, pH 4.5). Three days after STZ-injection, glucose concentration was measured in blood samples collected from the tail vein using BREEZE® 2 blood glucose monitoring system (Bayer Diabetes Care, USA). Only rats with blood glucose concentration higher than 270 mg/dl were considered diabetic and included in the STZ group. At the sacrifice, blood samples were collected for the quantification of plasma glucose (BREEZE® 2, Bayer Diabetes Care, USA) and hemoglobin A1C levels (A1CNow+®, Bayer Diabetes Care, USA).

2.3. Catheter implantation

Three weeks after diabetes induction, a silicon catheter was implanted into the lumbar subarachnoid space for intrathecal (i.t.) administrations. Animals were deeply anaesthetised with a mixture of ketamine and medetomidine (75 mg/kg of ketamine and 1mg/kg of medetomidine, i.p). Anesthesia was maintained during the surgical procedure with controlled levels of volatile anesthetic (isoflurane; 0.5-2%) in 60% oxygen/air mixture. A laminectomy was performed at T_8 –T_9 levels and a sterile silicone catheter (length: 13cm, inside diameter: 0.31mm, outside diameter: 0.64mm) (60-011-01; Helix Medical Europe SE & Co.KG, Germany) was introduced into the subarachnoid space and advanced 2.5–3 cm caudally, until the tip of the catheter was positioned at L_4 – L_5 spinal levels. The other end of the intrathecal catheter was sealed, externalized and fixed to the back of the neck. Animals were allowed to recover for a week. At the end of experiments, during the dissection procedure, the position of the catheter tip was verified and only the animals with the catheter correctly positioned at L_4 – L_5 levels were included in the study.

2.4. Behavioural evaluation of mechanical nociception

Four weeks after diabetes induction, mechanical nociception was evaluated by paw-pressure test (Randall-Selitto test, Ugo-Basile, Comerio, Italy) in STZ-diabetic and age-matched control rats receiving intrathecal infusions of saline (n=5) or the 5-HT3R antagonist (Y-25130 hydrochloride, n=5) in a dose of 30 fmol. The selection of 5-HT3R antagonist and respective dose was performed in accordance with a previous study using the same administration route (Guo et al., 2014). The mechanical force, in grams, that induced hindpaw withdrawal was recorded before intrathecal injection, at 30 min, 2h, 4h, 6h and 24h post injection. A 25 µl Hamilton syringe was attached to the silicon catheter and 25 µl of saline or 5-HT3R antagonist was slowly injected, followed by a flushing with saline to guarantee that all the solution was injected. Intrathecal injections were performed under light anaesthesia (1.5-3% isoflurane in 60% oxygen/air mixture).

In order to evaluate GABA involvement in 5-HT3R mediated pain modulation, additional groups of STZ-diabetic animals received intrathecal administrations of Y25310 (n=5) or saline (n=5), followed by an intrathecal administration of the $GABA_A$ receptor agonist muscimol (0.3 ug), 30 min before the maximum peak effect of Y25310 (3.5 h after Y25310 or saline administration)**.** The mechanical nociception was evaluated 30 min after muscimol injection. A 10 µl Hamilton syringe was attached to the silicon catheter and 10 µl of muscimol was slowly injected, followed by flushing with saline to guarantee that all the solution was completely delivered. The dose of intrathecal muscimol used was chosen in accordance with previous studies using the same administration route (Hwang and Yaksh, 1997; Jolivalt et al., 2008).

2.5. Immunohistochemistry

To evaluate the effects of 5-HT3R on spinal sensitization, additional groups of control and STZ-diabetic animals were treated with saline or Y-25130 hydrochloride, as described in 2.4, and sacrificed at the time-point showing the maximum effect of treatment. The animals were deeply

anaesthetized with a ketamine/medetomidine mixture (75 mg/kg of ketamine and 1mg/kg of medetomidine, i.p) and transcardially perfused with 250 ml of calcium free Tyrode's solution, followed by 1000 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PFA). The spinal segments L4–L5 were removed, post-fixed in 4% PFA for 2–4h and transferred to 30% sucrose in 0.1 M PBS overnight at 4 °C. Coronal sections, 40 µm thick, were obtained using a freezing microtome. One in each 4 sections was immunoreacted against the subunit A of 5-HT3 receptor (5-HT3RA) or phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK1/2, marker of nociceptive activity) (Ji et al., 1999), using the avidin–biotin–peroxidase method. The sections were initially treated with 1% hydrogen peroxidase for 20 min in order to inhibit the activity of endogenous peroxidase followed by an incubation period of 2h in a blocking solution of 10% normal horse serum (NHS) in 0.3% Triton X 25% in PBS (PBST) with 0.1 M glycine. The sections were then incubated two overnights at 4ºC in goat anti-5HT3RA (1:250; ref: AP16518PU-N; Acris Antibodies) or mouse anti-pERK1/2 (1:1000; ref: ab50011; Abcam) primary antibodies diluted in PBST with 2% NHS. After being washed in PBST with 2% NHS, the sections were incubated in biotinylated horse anti-goat immunoglobulin for 5-HT3RA (Vector Laboratories) and horse anti-mouse immunoglobulin for pERK1/2 (Vector Laboratories), all diluted at 1:200 in PBST with 2% NHS, for 1h, at room temperature. Sections were then washed in PBST, incubated for 1 h in the avidin–biotin complex (Vectastain, Vector Laboratories), and stained with diaminobenzidine (10 mg diaminobenzidine in 20 ml of Tris–HCl 0.05 M, pH 7.6 solution with 5 µl of 30% hydrogen peroxide). Sections were mounted on gelatin-coated slides, air-dried, and coverslipped with Eukit medium. Photomicrographs of sections were obtained by using an optical light microscope coupled with a high-resolutions digital camera (Axioskop 40; Zeiss, Hertfordshire, UK). Acquisition conditions (objective amplification, light intensity, contrast and hue) were maintained constant in all photomicrographs captions. The 5-HT3RA expression levels were quantified bilaterally in lamina I-V by imunolabelling densitometric analysis (6-8 sections per animal; n=4 *per* experimental group), using Image J software. For pERK1/2 quantification the numbers of pERK1/2-immunoreactive (pERK1/2-IR) cells were counted

bilaterally in laminae I–V (9-10 sections per animal; n=3-5 *per* experimental group). Delamination of the spinal cord was performed according to The Rat Brain Atlas (Paxinos and Watson, 2007). All analysis were performed by a blinded experimenter.

2.6. Western Blotting

For western blot experiments, control and STZ-diabetic animals received intrathecal infusions of saline or Y-25130 hydrochloride, as described in 2.4, and were sacrificed by decapitation under deep anaesthesia (75 mg/kg of ketamine and 1mg/kg of medetomidine, i.p) at the peak maximum Y25130 effects (n=3-5 *per* experimental group). The spinal L4-L5 segments were immediately removed and frozen at -80 °C. The spinal segments were then homogenized in a lysis buffer (20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, 1% Triton X-100) enriched with proteinase and phosphatase inhibitor cocktails (1:100; Sigma-Aldrich). Homogenates were centrifuged at 21100g for 20 min at 4 °C and the supernatants were collected. The protein concentration was quantified using Bradford Assay (Bradford Reagent; ref: #500-0205; Bio-Rad), using serial diluted bovine serum albumin (BSA) solutions as standards. Samples were heated at 100ºC for 5 min and 50µg protein of each sample was loaded onto 12% SDS-polyacrylamide gel, separated by electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 5% BSA for 1 h and incubated overnight at 4ºC with mouse anti-pERK1/2 (1:5000; ref: ab50011; Abcam), rabbit anti-ERK1/2 (1:1000, ref: 04/2010; Cell Signalling Technology), goat anti-5HT3RA (1:500; ref: AP16518PU-N; Acris Antibodies), mouse anti-α-Tubulin (1:10000; ref: T5168; Sigma-Aldrich), mouse anti-glial fibrillary acidic protein (GFAP) (1:1000; ref: G3893; Sigma-Aldrich), rabbit anti-ionized calcium binding adaptor molecule 1 (IBA1) (1:1000; ref: 019-19741; Wako) and rabbit anti-β-Actin (1:1000; ref: sc-130657; Santa Cruz Biotechnology) primary antibodies. The membranes were washed and incubated for 1h in horseradish peroxidase-conjugated secondary antibodies anti-mouse for pERK1/2, GFAP and α-Tubulin (1:10000; ref: NA931VS; Amersham), anti-goat for 5HT3RA (1:10000; ref: sc-2020, Santa Cruz Biotechnology) or anti-rabbit for IBA-1 and

β-Actin (1:10000; ref: NA934VS; Amersham). The pERK1/2/total ERK1/2 ratio was calculated to determine the phosphorylated fraction of ERKs. The signal was detected using a sensitive chemiluminescence reagent (Clarity Western ECL, ref: #170-5061; Bio-Rad). The GFAP and IBA-1 expression was used as astroglia and microglia markers, respectively (Ohsawa et al., 2004; Brahmachari et al., 2006). The α-Tubulin and β-actin were used as internal standards. Images were captured using a ChemiDoc XRS system (Bio-Rad) and the densitometric analysis of the bands was performed using Image Lab 5.1 software (Bio-Rad).

2.7. Drugs

Streptozotocin was purchased from Sigma-Aldrich, Spain. 5-HT3R antagonist Y-25130 hydrochloride and $GABA_A$ receptor agonist muscimol were purchased from TOCRIS Bioscience, UK.

2.8. Antibodies specificity

The specificity of each primary antibody was previously demonstrated (Kanazawa et al., 2002; Ralph et al., 2006; Wang and Hatton, 2009; Fei et al., 2011; Kaur and Tikoo, 2013; Uslu et al., 2014). Control experiments were performed by omission of primary or secondary antibodies. Additionally, for western blot experiments, the specificity of the primary antibodies was demonstrated by the detection of bands in the expected molecular weight.

2.9. Statistical analysis

The statistical analysis of data was performed using GraphPad PRISM version 6.0. The results of the behavioral evaluation of the effects of intrathecal administration of Y25130 on mechanical nociception were compared using two-way analysis of variance (ANOVA) repeated measures, followed by Tukey post-hoc test for multiple comparisons. The results of the behavioral evaluation of the effects of muscimol and of immunohistochemistry and the western blots experiments were compared using one-way ANOVA,

followed by Tukey post-hoc test for multiple comparisons. Independent sample t-test was used to compare metabolic parameters and pretreatment behavioral data between STZ and control animals. Statistical significance was settled at $p < 0.05$. Results are expressed as mean ± standard error of the mean (s.e.m.).

3. Results

3.1. Metabolic characterization

Four weeks after the induction of diabetes, STZ-diabetic rats presented significantly increased blood glucose concentration (STZ: 512.19 ± 10.15 mg/dL; control: 121.3 ± 7.36 mg/dL; $p < 0.0001$) and hemoglobin A1C levels (STZ: 12.1 ± 0.25%; control: 4.8 ± 0.06% $p < 0.0001$), along with decreased body weights (STZ: 261.5 ± 6.55 g; control: 402.1 ± 5.98 g; $p < 0.0001$), when compared with age-matched control animals (Table S1), which is in accordance with previous reports using the same animal model (Courteix et al., 1993; Calcutt, 2004; Morgado and Tavares, 2007) and support the installation of diabetes.

3.2. Effect of i.t. administration of 5-HT3R antagonist on mechanical nociception

Four weeks after diabetes onset, STZ-diabetic rats developed mechanical hyperalgesia (Fig 1) , as demonstrated by the significantly lower pretreatment paw withdrawal threshold (PWT) of STZ-diabetic animals when compared with control animals (STZ: 59.8 ± 3.77 g; control: 103.0 ± 2.49 g; $p < 0.0001$). Intrathecal delivery of Y25130 significantly increased the PWT in STZ-diabetic rats, showing an antinociceptive effect of Y25130 during DNP. The antinociceptive effects of Y25130 in STZ-diabetic animals were evident at 0.5h (STZ+Saline: 59.8 ± 5.05 g; STZ+Y25130: 88.4 ± 2.26 g; $p < 0.001$) and had a maximum peak at 4h (STZ+Saline: 61.0 ± 3.22 g; STZ+Y25130: 109.8 ± 7.39 g; $p < 0.0001$), returning to pretreatment values 6h post-administration (STZ+Y25130 pretreatment: 62.2 ± 3.38 g; STZ+Y25130: 71.4 ± 3.29 g; $p >$

0.05). The administration of Y25130 had no effect in the mechanical response thresholds of control rats in any time-point evaluated (control+Y25130 pretreatment: 101.8 ± 4.80 g; control+Y25130 at 0.5h post-injection: 96.6 ± 9.22 g, control+Y25130 at 4h post-injection: 102.8 ± 6.62, control+Y25130 at 6h post-injection: 95.2 ± 4.55 g g; $p>0.05$) (Fig 1).

3.3. Effect of i.t. administration of 5-HT3R antagonist on spinal ERK1/2 activation

The STZ-diabetic rats receiving saline infusions presented a significantly higher number of pERK1/2- IR cells when compared with control animals (STZ+Saline: 657.9 ± 37.42; control+Saline: 306.9 ± 58.63, $p < 0.01$) (Fig 2). Administration of Y25130 to STZ-diabetic animals significantly reduced spinal ERK1/2 activation (Fig 2), as demonstrated by the significantly lower number of pERK1/2-IR cells observed in the spinal sections of Y25130-treated STZ-animals (STZ+Saline: 657.9 ± 37.42; STZ+Y25130: 417.0 ± 41.27, $p < 0.05$) (Fig 2a and c-f). The treatment had no effects in the number of pERK1/2-IR cells in the spinal cord of control rats (control+Saline: 306.9 ± 58.63; control+Y25130: 293.1 ± 44.67, $p > 0.05$). Western blotting quantification of pERK1/2 and ERK1/2 expression in spinal homogenates from STZ-diabetic and control animals treated with saline or Y25130 also showed that administration of Y25130 significantly reduced spinal ERK1/2 activation in STZ-diabetic rats, as demonstrated by the significantly lower phosphorylated fraction of ERK1/2 content in STZ-diabetic rats treated with Y25130 (STZ+Saline: 4.0 ± 1.00; STZ+Y25130: 1.2 ± 0.49; control+Saline: 1.5 ± 0.05; control+Y25130: 1.2 ± 0.34, $p <0.05$) (Fig 2b).

3.4. Effect of i.t. administration of 5-HT3R antagonist on spinal glia activation

In order to evaluate if spinal 5-HT3R antagonism during DNP interferes with glia activity, we evaluate the effect of spinal 5-HT3R inhibition on spinal GFAP and IBA-1 expression levels. The expression of GFAP was significantly lower in STZ-diabetic rats than in controls animals (STZ+Saline: 0.04 ± 0.012;

control+Saline: 0.92 ± 0.085; p <0.01) (Fig S1b and c). The expression levels of IBA-1 were significantly higher in STZ-animals when compared with control rats (STZ+Saline: 1.9 ± 0.08; control+Saline: 1.1 ± 0.05; p <0.01) (Fig S1a and c). The spinal 5-HT3R inhibition did not affect the expression levels of GFAP (STZ+Saline: 0.04 ± 0.012; STZ+Y25130: 0.04 ± 0.006; p > 0.05; control+Saline: 0.92 ± 0.085; control+Y25130: 0.96 ± 0.21, p >0.05) and IBA-1 (STZ+Saline: 1.9 ± 0.08; STZ+Y25130: 1.8 ± 0.12; p >0.05; control+Saline: 1.1 ± 0.05; control+Y25130: 1.2 ± 0.12; p >0.05), neither in STZ-diabetic nor in control rats (Fig S1).

3.5. Expression of 5-HT3RA at the spinal dorsal horn

Since 5-HT3R inhibition had differential effects on nociceptive responses of STZ-diabetic and control animals, we hypothesized that the selective 5-HT3R-mediated pain facilitation during DNP could be explained by a possible change in the expression of the receptor induced by diabetes. Densitometric immunolabelling analysis of spinal 5-HT3RA expression did not reveal significant differences between the STZ-diabetic and control rats (STZ+Saline: 46.0 ± 1.90; STZ+Y25130: 39.8 ± 2.92; control+Saline: 40.6 ± 4.77; control+Y25130: 43.6 ± 2.94; p > 0.05) (Fig S2a and c-f). These findings were corroborated by western blotting analysis of the receptor expression (STZ+Saline: 0.3 ± 0.08; STZ+Y25130: 0.5 ± 0.14; control+Saline: 0.3 ± 0.02; control+Y25130: 0.4 ± 0.12; p > 0.05) (Fig S2b).

3.6. Effect of i.t. administration of $GABA_AR$ agonist on the effect of spinal 5-HT3R inhibition

Muscimol administration prevented the antinociceptive effect elicited by 5-HT3R inhibition in STZ-diabetic rats, as showed by the reduction in PWT to levels of pretreatment in muscimol+Y25130-treated STZ-animals (STZ+Y25130 pretreatment: 60.6 ± 2.46 g; STZ+Y25130 at 4h post-injection: 109.8 ± 7.39 g; STZ+Y25130+Muscimol at 4h post-injection: 57.0 ± 6.43 g, p<0.001 in STZ+Y25130 at 4h post-injection vs the other groups) (Fig 3). Muscimol had no effects in the mechanical response thresholds of STZ-

diabetic animals receiving saline infusions (STZ+Saline pretreatment: 61.0 ± 3.22 g; STZ+Saline+Muscimol at 4h post-saline injection: 56.0 ± 7.10 g, $p>0.05$) (Fig 3).

Discussion

By using a pharmacological approach to inhibit the 5-HT3R at the spinal cord in a validated model of DNP, the present study is the first to demonstrate that spinal 5-HT3R activation is involved in pain facilitation and contributes to spinal sensitization through the activation of ERK1/2 pathways during DNP. The present study also provides new insights into the mechanisms underlying 5-HT3R-spinal nociceptive modulation in chronic pain by showing that 5-HT3R pronociception is mediated by spinal GABAergic signalling.

The development of persistent pain appears to be dependent, in part, upon increased drive of RVM-arising 5-HT descending pathways, which leading to activation of 5-HT3Rs at the spinal level, seems to facilitate pain transmission during chronic pain (Suzuki et al., 2002; Dogrul et al., 2009). The pain behavior detected in the second-phase of the formalin test, but not that observed in the first-phase, has been reported to be significantly reduced in mice lacking the subunit A of 5-HT3R and in animals receiving intrathecal administrations of 5-HT3R antagonists, suggesting an involvement of 5-HT3R in pain chronification (Oyama et al., 1996; Zeitz et al., 2002). However, some contradictory evidences exist in what concern the role of spinal 5-HT3R in pain modulation, with studies demonstrating that spinal 5-HT3R elicits antinociceptive effects in acute pain (Glaum et al., 1990; Alhaider et al., 1991). These findings point for a possible shift in the role of spinal 5-HT3R in chronic pain conditions. Our data show that inhibition of spinal 5-HT3R by intrathecal administration of a selective 5-HT3R antagonist reverted the mechanical hyperalgesia in STZ-diabetic rats. Previous studies using STZ-diabetic rats reported an increased activation of serotonergic neurons at the RVM along with higher spinal serotonin contents during DNP (Morgado et al., 2011b), suggesting an increased RVM descending serotoninergic drive during this chronic pain condition. This may lead to overactivation of spinal 5-HT3R,

which, taking into account the pain facilitatory role here reported, is likely to contribute to mechanical hypersensitivity associated to DNP. No differences were observed in the spinal expression of 5-HT3RA in STZ-diabetic rats, which reinforces the hypothesis that 5-HT3R-mediated pain facilitation during DNP is likely to be caused by increased serotonin bioavailability rather than due to changes in the expression of the receptor. Studies also showed unaltered 5-HT3R expression in other chronic pain conditions (Rahman et al., 2009). Our findings along with the lack of antinociceptive effect of a 5-HT3R antagonist in an animal model of traumatic neuropathy, where increased descending serotoninergic drive was not verified (Peters et al., 2010), clearly point to the important role of enhanced activity of descending serotoninergic pathways in spinal 5-HT3R pain facilitation during DNP.

Increasing evidences show that extracellular signal-regulated kinases 1 and 2 (ERK1/2) expressed in the spinal cord are involved in nociceptive processing and spinal sensitization (Gao and Ji, 2009; Han et al., 2011). In fact, ERK1/2 are strongly activated in the spinal dorsal horn following peripheral inflammation and tissue/nerve injury and the pharmacological blockade of this activation reduces the hypersensitivity otherwise observed in these experimental models (Ji et al., 1999; 2002). This activation of ERKs has been attributed to the hyperexcitatibility of spinal dorsal horn neurons evoked by increased peripheral barrage. This assertion, while reasonable, discards the possible contribution of other dorsal horn inputs that are believed to regulate dorsal horn excitability, namely the inputs arising from descending modulatory pathways. Increased activation of ERK1/2 in STZ-diabetic rats was observed in the present study, in agreement with data from a previous study (Daulhac et al., 2006*)*. In addition, our findings demonstrate that the i.t. administration of 5-HT3R antagonist reverted the increased spinal ERK1/2 activation in STZ-diabetic rats, pointing for a role of 5-HT3R-mediated descending serotoninergic facilitation in spinal sensitization during DNP. Consistent with our data, previous studies demonstrated that the depletion of spinal 5-HT reduced the formalin evoked flinching and activation of spinal ERK1/2 pathways (Svensson et al., 2006*)*. Moreover, intrathecal administration of ondansetron (a 5-HT3 receptor antagonist) at doses that inhibited formalin-induced flinching also attenuated spinal ERK activation

(Svensson et al., 2006). Taken together these findings revealed that spinal ERKs activation requires the input from an excitatory serotoninergic pathway and is dependent on 5HT3R activity.

At the spinal cord, 5-HT3R expression seems to be restricted to some neuronal subpopulations and primary afferents terminals (Kia et al., 1995), with no reported expression in glial cells (Guo et al., 2014). The mechanisms underlying 5-HT3R-mediated spinal pain modulation remain elusive. Recently, a 5-HT3R-dependent neuronal-glial crosstalk was proposed as a possible mechanism underlying pain facilitation induced by the pharmacological activation of spinal 5-HT3R in healthy animals (Guo et al., 2014), with an i.t. injection of 5-HT3R agonist leading to increased activation of microglia and astroglia and the reversion of these effect by treatment with a 5-HT3R specific antagonist. In the present study no differences were detected in the activation of spinal microglia or astroglia upon i.t. administration of 5-HT3R antagonist in STZ-diabetic rats. Our results do not seem to support a role for glia in the pronociceptive effects of spinal 5-HT3R, at least in this chronic pain condition. Indeed, it is important to note that Guo et al. (2014) studied the role of glial cells in the effects of spinal 5-HT3R activation in an acute pain condition and no reports exist on the effects of chronic pain in this mechanism. In the present study we used an animal model of chronic pain that already presents altered spinal glial responses before i.t. administration of 5-HT3R antagonist (Daulhac et al., 2006; Tsuda et al., 2008; Wodarski et al., 2009; Morgado et al., 2011a). The absence of a normal functioning of glia signalling cascade during DNP can contribute to the lack of effects of 5-HT3R on glia activation here reported.

The interplay between 5-HT and GABA was shown to be crucial in spinal modulation of nociceptive transmission. The electrical stimulation of RVM neurons elicits GABA-mediated inhibitory post-synaptic potentials (IPSPs) in primate spinothalamic tract neurons (Giesler et al., 1981). Furthermore, the $GABA_A$-R agonist, muscimol, and 2-methyl 5-HT, a non-selective 5-HTR agonist, was shown to have similar effects on nociceptive spinal projection neurons firing elicited by excitatory amino acids (Lei and Wilcox, 1990). More recently, 5-HT3R expression was reported in an intrinsic GABAergic neuronal subpopulation of the spinal cord, and its activation on

these neurons was shown to enhance GABA release (Fukushima et al., 2009). This enhancement is likely to activate postsynaptic $GABA_AR$, which was shown to culminate in antinociception by inhibiting spinothalamic tract ascending neurons (Alhaider et al., 1991; Kawamata et al., 2003). If in normal conditions GABA seems to play a major inhibitory tone on spinal nociceptive transmission, several studies have been showing that persistent pain conditions, including DNP, are accompanied by a shift in the role of GABA from inhibitory to excitatory, due to the decrease of spinal potassium chloride co-transporter 2 (KCC2) expression (Jolivalt et al., 2008; Morgado et al., 2008). The KCC2 downregulation promotes an accumulation of intracellular chloride which causes an outflow of chloride ions upon the binding of GABA to $GABA_AR$, leading to neuronal excitation instead of inhibition and contributing to the spinal nociceptive hyperactivity observed in DNP (Morgado et al., 2011a). Our results show that the antinociceptive effects of i.t. administered 5-HT3R antagonist is abolished by i.t. delivery of muscimol, suggesting that the effects elicited by 5-HT3R antagonism are mediated by the reduction of $GABA_AR$ activation, probably due to a decrease in spinal GABA release.

In conclusion, the data gathered by the present study suggest that 5-HT3R-mediated increase in spinal GABAergic transmission, probably due to the overactivation of descending serotoninergic pathways, can mediate pain facilitation during DNP. This study provides new insights on the mechanisms underlying the contribution of spinal 5-HT3Rs to pain modulation during chronic pain. Accordingly, and attending to the already reported good tolerability and pharmacokinetic profile of 5-HT3Rs inhibitory drugs in the clinical practice (McCleane et al., 2003), the use of 5-HT3R antagonists may then be considered as a promising pharmacological approach in alleviating the mechanical hyperalgesia associated to diabetic neuropathy.

Author contributions

All authors participate in experimental conception and design. C. Morgado and I. Tavares supervised the experiments. M. Silva performed all the surgeries and behavioural tests. M. Silva and D. Martins performed

immunohistochemistry and western blot experiments. All authors discussed the results. M. Silva and D. Martins wrote the first drafts of the manuscript. C. Morgado and I. Tavares revised the manuscript. All authors have read and approved the final manuscript.

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Legends

Figure 1 - Effects of intrathecal administration of 5-HT3R antagonist on mechanical nociception. *Control (saline and Y25130) vs STZ-diabetic (Saline and Y25130); # STZ+ Saline vs all other groups; two symbols p<0.01, three symbols p<0.001, four symbols p<0.0001, by Two-Way ANOVA repeated measures followed by Tukey *post-hoc* test for multiple comparisons.

Figure 2 – Effects of intrathecal administration of 5-HT3R antagonist on spinal ERK1/2 activation. (a) Number of pERK1/2-labeled cells in lamina I-V in sections from L4-L5 spinal segments; (b) quantification by western blotting of pERK1/2 expression in the L4 – L5 spinal cord homogenates and representative blots; (c-f) representative photomicrographs of sections from spinal segments L4-L5 immunoreacted against pERK1/2 in Control+Saline (c), Control+Y25130 (d), STZ+Saline (e) and STZ+Y25130 (f). (e') Representative photomicrograph of high magnification of pERK1/2-labeled cells in lamina I-II of L4 spinal segment from STZ+saline rats. Scale bar in f = 500µm, scale bar in e'= 200µm. #STZ+saline vs control groups; *STZ+saline vs STZ+Y25130; one symbol p<0.05, two symbols p<0.01, by One-Way ANOVA followed by Tukey *post-hoc* test for multiple comparisons.

Figure 3 – Effects of intrathecal muscimol in the antinociception elicited by the inhibition of spinal 5-HT3R on STZ-diabetic rat. * STZ+Y25130 vs all other groups; three symbols p<0.001 by One-Way ANOVA followed by Tukey *post-hoc* test for multiple comparisons.

Table S1 - Blood glucose concentration, percentage of hemoglobin A1C and body weights of STZ-diabetic and control animals.

Figure S1 – Effects on intrathecal administration of 5-HT3R antagonist on the spinal expression levels of GFAP and IBA-1. (a) Quantification by western blotting of GFAP expression in the L4 – L5 spinal cord homogenates and representative blots; (b) quantification by western blotting of IBA-1 expression in the L4 – L5 spinal cord homogenates and representative blots. * Control vs

STZ groups; two symbols $p<0.01$, by One-Way ANOVA followed by Tukey *post-hoc* test for multiple comparisons.

Figure S2 – Analysis of 5-HT3R subunit A (5-HT3RA) expression. (a) Immunolabelling optical density in lamina I-V for 5-HT3RA; (b) quantification by western blotting of 5-HT3RA expression in the L4 – L5 spinal cord homogenates and representative blots; (c-f) representative photomicrographs of sections from spinal segments L4-L5 immunoreacted against 5-HT3RA in Control+Saline (c), Control+Y25130 (d), STZ+Saline (e) and STZ+Y25130 (f). Scale bar: 500μm.

Figures

Figure 1.

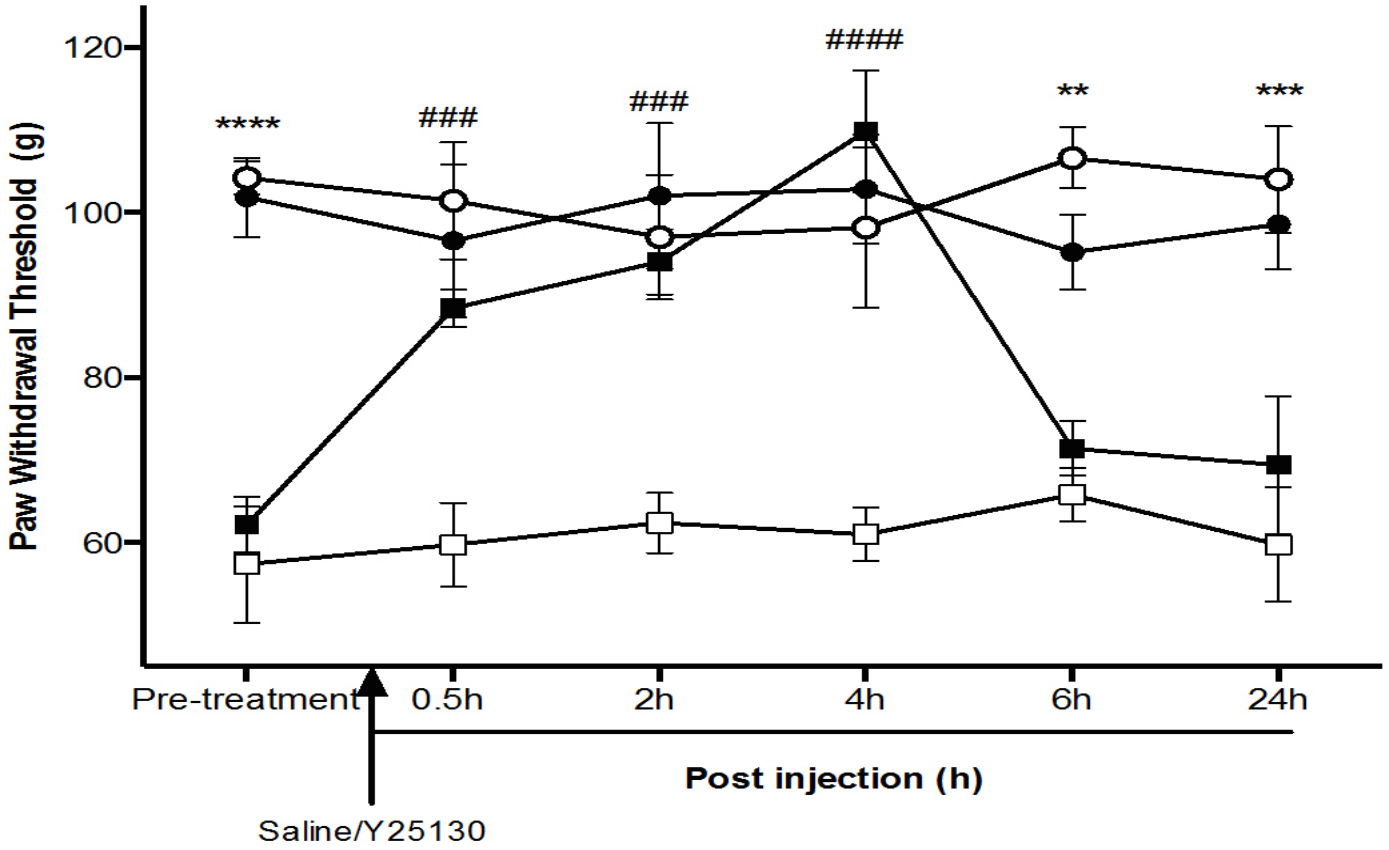


Figure 2.

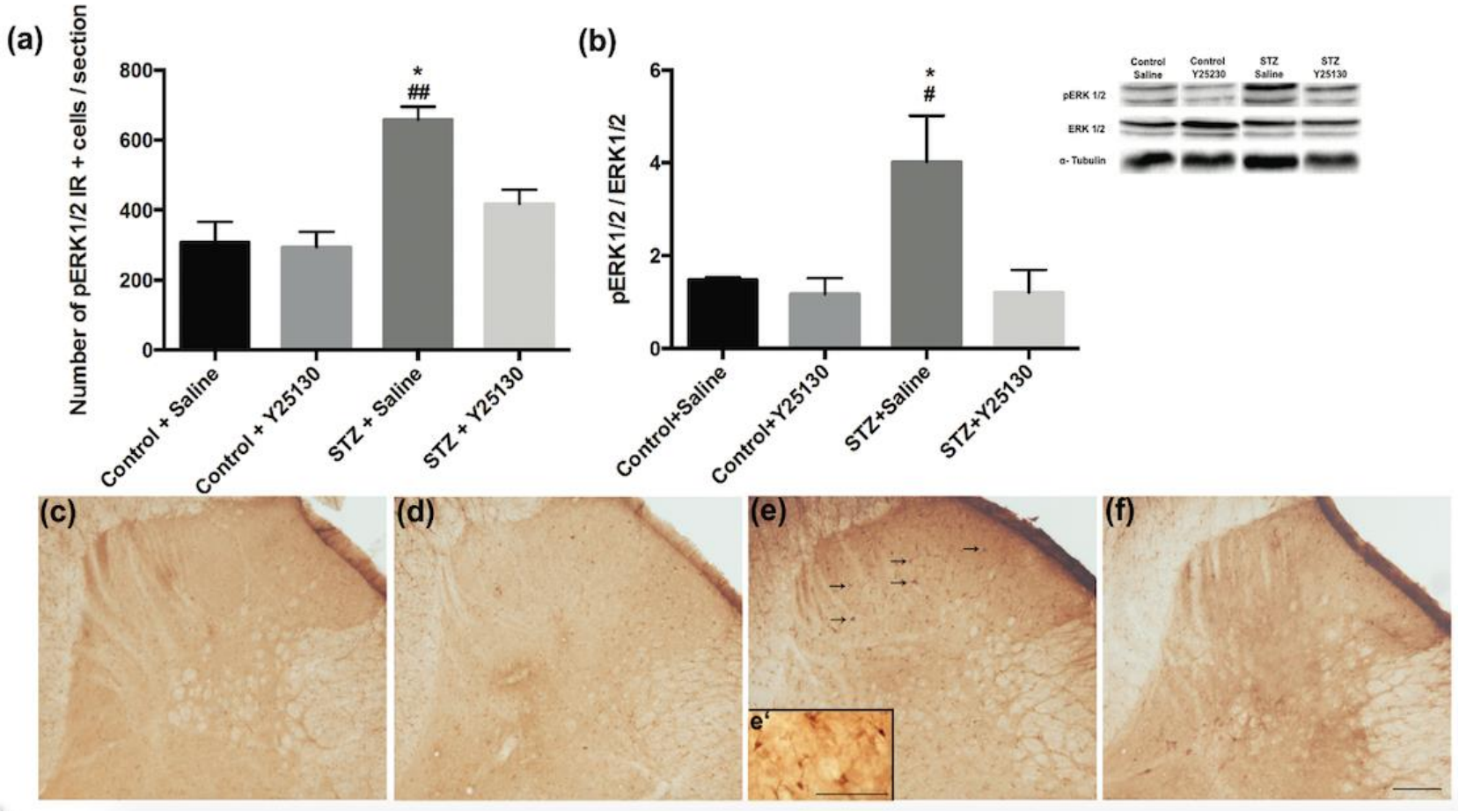

(a)
Number of pERK1/2 IR + cells / section
800
600
400
200
0
*
##
Control + Saline
Control + Y25130
STZ + Saline
STZ + Y25130
(b)
pERK1/2 / ERK1/2
6
4
2
0
*
#
Control+Saline
Control+Y25130
STZ+Saline
STZ+Y25130
Control Saline
Control Y25230
STZ Saline
STZ Y25130
pERK 1/2
ERK 1/2
α- Tubulin
(c)
(d)
(e)
e'
(f)

Figure 3.

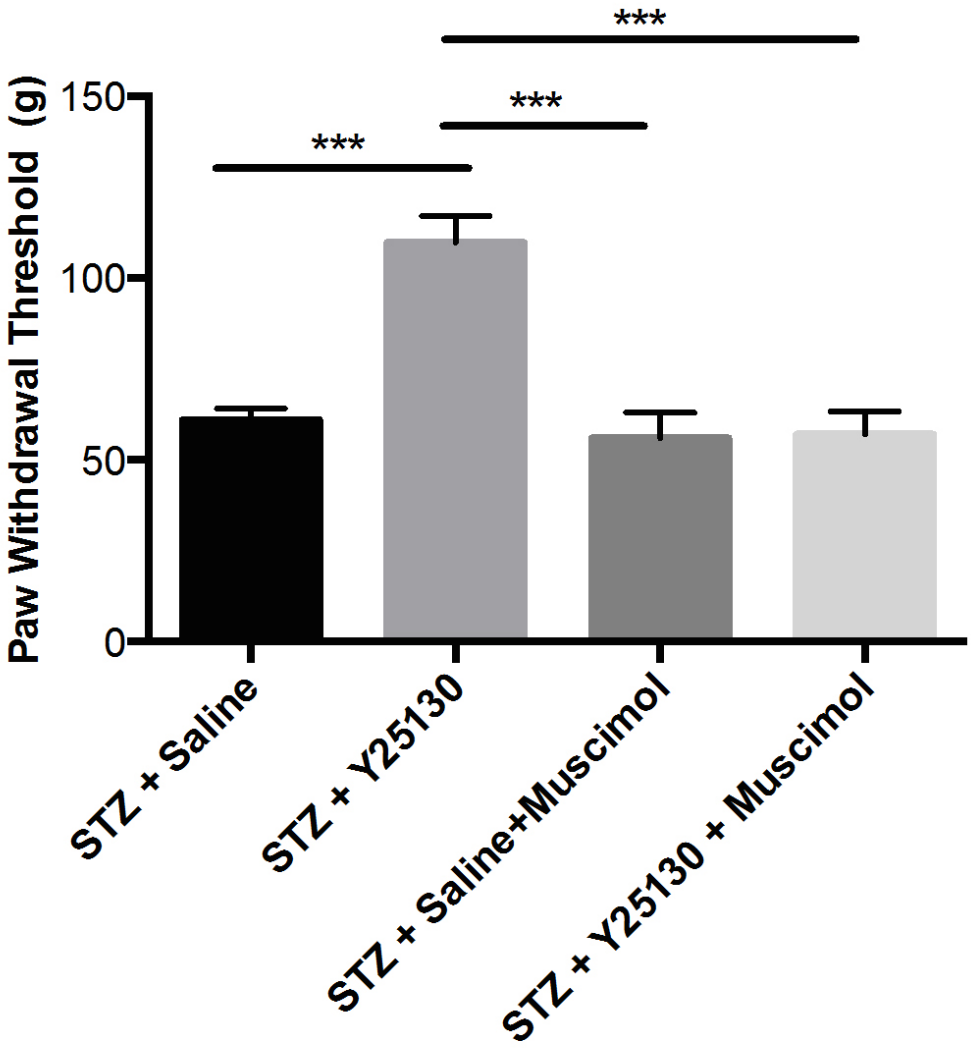

***
***
***
Paw Withdrawal Threshold (g)
150
100
50
0
STZ + Saline
STZ + Y25130
STZ + Saline+Muscimol
STZ + Y25130 + Muscimol

Table S1 - Blood glucose concentration, percentage of hemoglobin A1C and body weights of STZ-diabetic and control animals.

Parameters	STZ	Control
Blood Glucose concentration (mg/dl)	512.9 ± 10.15[a]	121.3 ± 7.36
Hemoglobin A1C (%)	12.1 ± 0.25[a]	4.8 ± 0.06
Body weight (g)	261.5 ± 6.55[a]	402.1 ± 5.98

Independent sample t test. [a]$p < 0.0001$.

Figure S1.

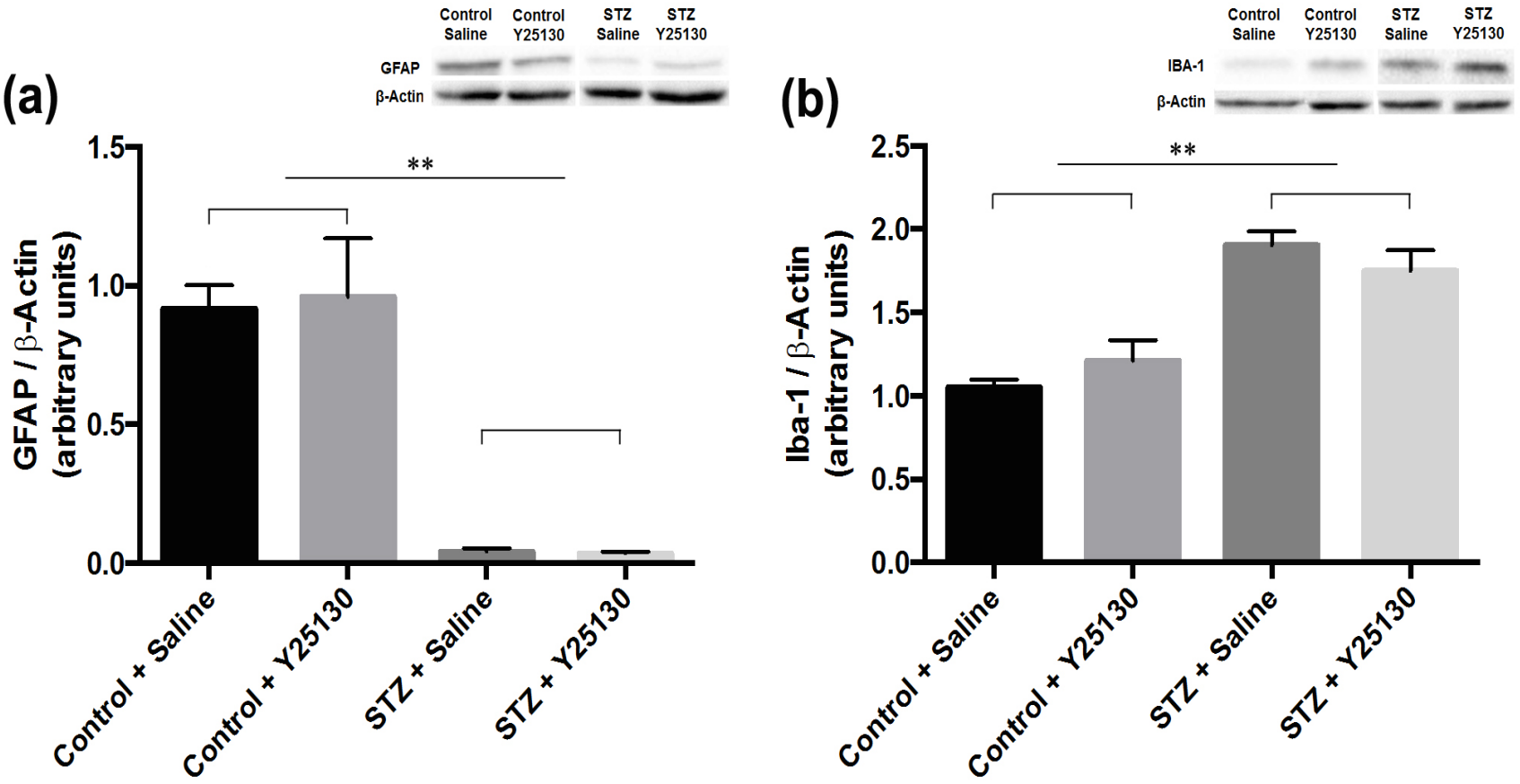
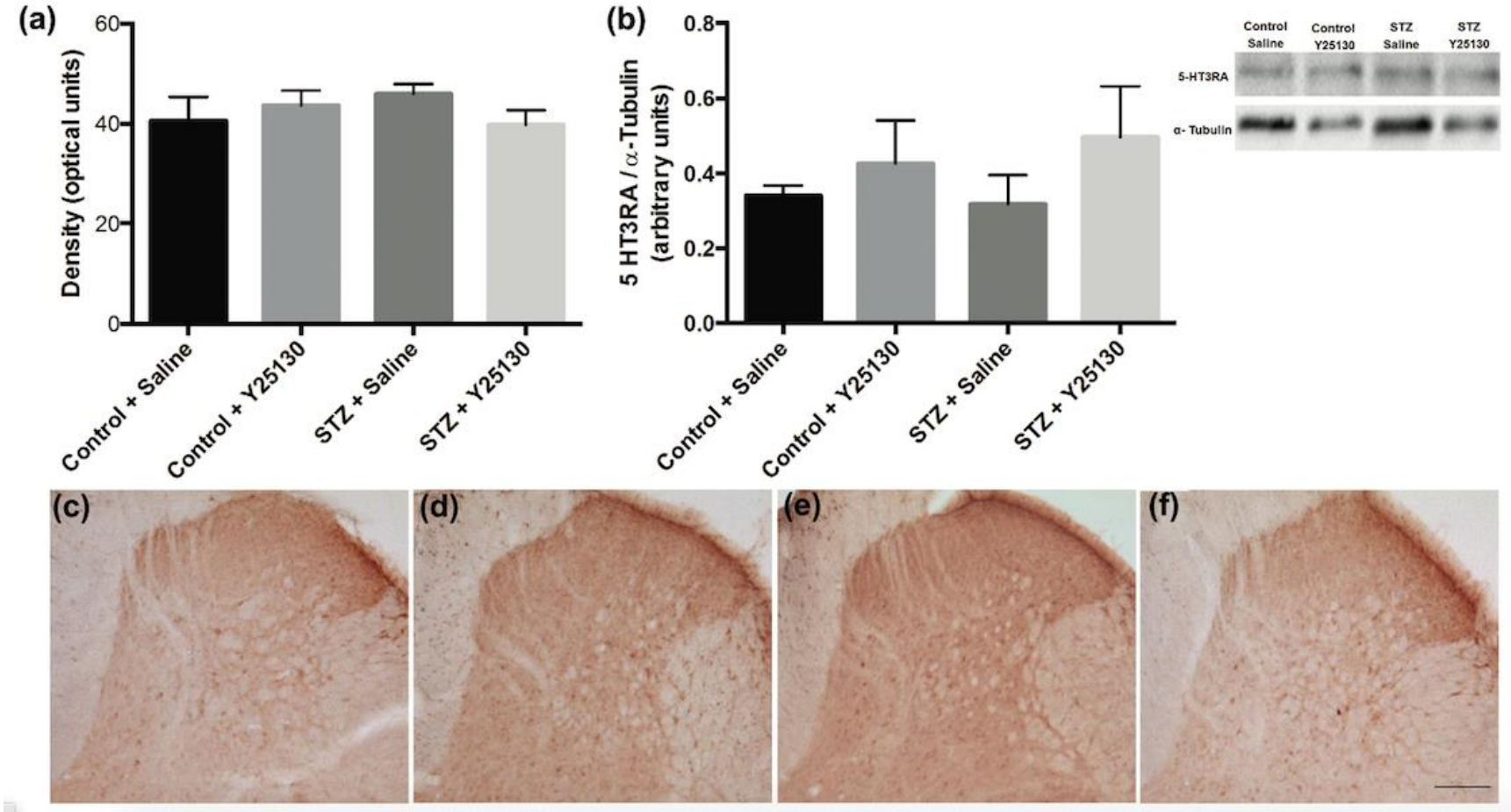

(a)
Control Saline
Control Y25130
STZ Saline
STZ Y25130
GFAP
β-Actin
GFAP / β-Actin (arbitrary units)
1.5
1.0
0.5
0.0
**
Control + Saline
Control + Y25130
STZ + Saline
STZ + Y25130
(b)
Control Saline
Control Y25130
STZ Saline
STZ Y25130
IBA-1
β-Actin
Iba-1 / β-Actin (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
**
Control + Saline
Control + Y25130
STZ + Saline
STZ + Y25130

Figure S2.


(a)
Density (optical units)
60
40
20
0
Control + Saline
Control + Y25130
STZ + Saline
STZ + Y25130
(b)
5 HT3RA / α-Tubulin (arbitrary units)
0.8
0.6
0.4
0.2
0.0
Control + Saline
Control + Y25130
STZ + Saline
STZ + Y25130
Control Saline
Control Y25130
STZ Saline
STZ Y25130
5-HT3RA
α- Tubulin
(c)
(d)
(e)
(f)

ANEXOS

ANEXO 1 - Authors Guidelines (European Journal of Pain)

Manuscript Structure and Word Count

1) Manuscript
    - Title page (see further details below)
    - Abstract (should not exceed 250 words, see further details below)
    - Text
        - o Introduction (no subheadings, should not exceed 500 words)
        - o Methods (or Literature Search Methods for Review Articles)
        - o Results
        - o Discussion and conclusions (should not exceed 1500 words)
    - Acknowledgements
    - Author contributions (see Section 6)
    - References (limited to 80 for original manuscripts)
    - Legends for illustrations and tables
2) Tables (to be uploaded as separate files)
3) Figures (to be uploaded as separate files)
4) Supporting material (additional material that will be published online-only, to be uploaded separately, see further details below)

Title Page

The title page should give:
1) The title of the article. Titles should be short and should not contain acronyms
2) A running head not exceeding 50 characters
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