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Progress in Neurobiology 193 (2020) 101846

Contents lists available at ScienceDirect

Progress in Neurobiology

journal homepage: www.elsevier.com/locate/pneurobio

Original Research Article

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ARTICLE INFO

Keywords:
Neuropathic pain
Cognitive deficit
5-HT$_6$ receptor
Constitutive activity
mTOR
Inverse agonist

ABSTRACT

Chronic neuropathic pain is a highly disabling syndrome that is poorly controlled by currently available analgesics. Here, we show that painful symptoms and associated cognitive deficits induced by spinal nerve ligation in the rat are prevented by the administration of serotonin 5-HT$_6$ receptor inverse agonists or by the mTOR inhibitor rapamycin. In contrast, they are not alleviated by the administration of 5-HT$_6$ receptor neutral antagonists. Likewise, activation of mTOR by constitutively active 5-HT$_6$ receptors mediates allodynia in oxaliplatin-induced peripheral neuropathy in rats but not mechanical nociception in healthy rats. Furthermore, both painful and co-morbid cognitive symptoms in neuropathic rats are strongly reduced by intrathecal delivery of a cell-penetrating peptide that disrupts 5-HT$_6$ receptor/mTOR physical interaction. Collectively, these findings demonstrate a deleterious influence of non-physiological mTOR activation by constitutively active spinal 5-HT$_6$ receptors upon painful and cognitive symptoms in neuropathic pains of different etiologies. They suggest that targeting the constitutive activity of 5-HT$_6$ receptors with inverse agonists or disrupting the 5-HT$_6$ receptor/mTOR interaction might be valuable strategies for the alleviation of neuropathic pain and cognitive co-morbidities.

1. Introduction

Neuropathic pains refer to an heterogeneous group of pains arising as a direct consequence of a lesion or disease affecting the somatosensory system (Treede et al., 2008). Neuropathic pains affect 5–10 % of the general population depending on the country and the screening tools used (Bouhassira et al., 2008; Chenaf et al., 2018; van Hecke et al., 2014). Due to their severity and chronicity, neuropathic pains often coexist with co-morbid symptoms such as anxiety, depression, sleep disturbances and cognitive deficits (Radat et al., 2013). Chronic neuropathic pain and co-morbidities are especially problematic because of the limited efficacy of first-line treatments. For instance, the number of patients who needed to be treated (NNT) to obtain a 50 % pain relief in one patient is 3.6 for the tricyclic antidepressant amitriptyline, one of reference treatments in neuropathic pain (Finnerup et al., 2015). Furthermore, reference treatments induce frequent adverse effects, with a safety profile, evaluated by the number needed to harm (number of patients who need to be treated for one patient to drop out because of adverse effects), in the range of 11.8–13.9 (Finnerup et al., 2015). Collectively, these observations underscore the need for new therapeutic strategies for the treatment of neuropathic pain.

Abbreviations: AUC, area under the curve; CIPN, chemotherapy-induced peripheral neuropathy; CPPQ, (*S*)-1-[(3-chlorophenyl)sulfonyl]-4-(pyrrolidine-3-yl-amino)-1H-pyrrolo[3,2-c]quinoline dihydrochloride; IIQ, 4-{[5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indol-1-yl]sulfonyl}isoquinoline dihydrochloride; MPE, maximal possible effect; NNT, number needed to treat; NOR, novel object recognition; OXA, oxaliplatin; PZ-1388, 2-(3-fluorophenyl)-1-[(3-chlorophenyl)sulfonyl]-N-(piperidin-4-yl)-1H-pyrrole-3-carboxamide hydrochloride; RM, repeated measure; SNL, spinal nerve ligation; SNRI, serotonin-noradrenaline reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant

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https://doi.org/10.1016/j.pneurobio.2020.101846
Received 17 December 2019; Received in revised form 6 May 2020; Accepted 2 June 2020
Available online 05 June 2020

Pain processing at the spinal level is finely controlled by serotonergic projections originating from the midbrain and brain stem (Millan, 2002). Given the multiplicity of serotonin receptors expressed in dorsal spinal cord and the resistance of neuropathic pain to selective serotonin reuptake inhibitor (SSRI) antidepressants, one possibility is that certain serotonin receptors induce pro-algesic effects. These include the 5-HT_6 receptor, which is expressed in excitatory interneurons of the dorsal spinal cord, especially in a region below lamina II inner to lamina IV outer called "low-threshold mechanoreceptor-recipient zone" and involved in tactile perception (Abraira et al., 2017). The 5-HT_6 receptor is coupled to the Gs-adenylyl cyclase pathway and stimulates cAMP production, which leads to depolarization of neurons. Consistent with these excitatory effects, 5-HT_6 receptor blockade with arylsulfonamide derivatives of pyrazolo[3,4]pyridine-7-ones and 1-aryl-5-isopropyl-pyrazoles or SB258585 produces anti-allodynic or anti-hyperalgesic effects in rodent models of traumatic (Devegowda et al., 2013; Hong et al., 2017; Pineda-Farias et al., 2017) and metabolic (Sari et al., 2019) neuropathic pain. 5-HT_6 receptor blockade also improves cognition in a wide range of cognitive impairment paradigms in rodents (Codony et al., 2011; Hirst et al., 2006; Loiseau et al., 2008; Vanda et al., 2018; Zajdel et al., 2016). Several 5-HT_6 receptor antagonists have entered clinical trials for the indication of cognitive deficits in various neuropsychiatric disorders, including schizophrenia and dementia (Khoury et al., 2018), underscoring their potential for the treatment of cognitive co-morbidities associated to neuropathic pain. An important feature of the 5-HT_6 receptor is its high level of constitutive activity, which was established not only for recombinant receptors expressed in transfected cells but also for native receptors present in cultured neurons or mice brain (Deraredj Nadim et al., 2016; Duhr et al., 2014; Grychowska et al., 2016; Kohen et al., 2001). Whether 5-HT_6 receptor constitutive activity contributes to neuropathic pain and cognitive co-morbidities and whether 5-HT_6 receptor inverse agonists could be useful for their clinical management have never been explored.

Using proteomics strategies, we previously showed that 5-HT_6 receptors physically interact with several proteins of the mechanistic Target Of Rapamycin (mTOR) pathway (Meffre et al., 2012). Further studies revealed that non-physiological activation of mTOR by prefrontal 5-HT_6 receptors underlies cognitive deficits in rodent development models of schizophrenia (Meffre et al., 2012). Interestingly, an increasing body of evidence indicates a role of mTOR signaling in neuropathic pain sensitivity: central administration of the mTOR inhibitor rapamycin reduces mechanical hypersensitivity in spared nerve injured rats (Geranton et al., 2009) and mice (Obara et al., 2011), as well as mechanical allodynia and thermal hyperalgesia in rats with impact-induced spinal cord injury (Wang et al., 2016). Rapamycin administration also attenuates mechanical allodynia induced by chronic nerve constriction injury in mice (Zhang et al., 2013), reduces mechanical allodynia and cold hypersensitivity in bortezomib-induced peripheral neuropathy in rats (Duan et al., 2018) and mechanical hyperalgesia in streptozotocin (STZ)-induced diabetic neuropathy (He et al., 2019).

In light of these observations, we investigated, in the present study, the role of 5-HT_6 receptor constitutive activity and mTOR signaling under the control of constitutively active 5-HT_6 receptors in neuropathic pain and co-morbid cognitive symptoms induced by spinal nerve ligation (SNL) or oxaliplatin (OXA) administration in the rat. We show in both models that activation of mTOR by constitutively active spinal 5-HT_6 receptors mediates allodynia and co-morbid cognitive symptoms. These findings suggest that 5-HT_6 receptor inverse agonists currently under clinical evaluation for the treatment of cognitive impairment in dementia, and mTOR inhibitors already used to prevent graft rejection in transplanted patients or for the treatment of breast and kidney cancer, might be repositioned for the alleviation of neuropathic pains of various etiologies and cognitive co-morbidities.

2. Methods and materials

2.1. Animals

All animal procedures described here were reviewed and approved by the Auvergne Animal Experiment Ethics Committee, CE2A and by the French Ministry of Higher Education and Innovation (authorization N° 2018111617333273V5) and conducted in accordance with the NC3R ARRIVE rules (Kilkenny et al., 2010). Sprague-Dawley adult male rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Male rats were chosen for all behavioral experiments to avoid any influence of ovarian hormone variations due to the estrus cycle on pain sensitivity (Fischer et al., 2008).

The transgenic mouse line C57BL/6 *knock-in* 5-HT_6-GFP generated by the Institut Clinique de la Souris (www.ics-mci.fr), was described elsewhere (Deraredj Nadim et al., 2016). The animals were housed under a 12-h light/dark cycle at 21 ± 2 °C and 55 ± 10 % humidity with *ad libidum* access to food and water in an animal facility free of specified pathogenic organisms.

Experimenters were blinded to the treatment. Treatments were randomized and administered according to the method of blocks in order to assess their effect in the same conditions. Different animals were used in each experiment. At the end of the experiments, the animals were euthanized by progressive carbon dioxide inhalation (10–30 %/min).

2.2. Animal models of neuropathic pain

Traumatic neuropathic pain was induced by SNL. The surgery was performed as previously described (Kim and Chung, 1992). Rats (200 ± 20 g) were anesthetized by i.p. injection of xylazine (10 mg/kg) and ketamine (75 mg/kg). Under aseptic conditions, the left L5 spinal nerve was ligated with a nonabsorbable 5 − 0 braided silk thread. Sham surgery was performed in an identical manner except for the nerve ligation. At the end of the surgery, the rats received a s.c. injection of meloxicam (non-steroidal anti-inflammatory drug) to reduce the post-surgery pain and inflammation.

Chemotherapy-induced peripheral neuropathy (CIPN) model was induced by a single i.p. injection of oxaliplatin (OXA, 6 mg/kg) dissolved in a 5 % glucose solution, as previously described (Ling et al., 2007).

2.3. Behavioral tests

Tactile allodynia was assessed using the von Frey hair test (Chaplan et al., 1994). Animals were habituated to the testing environment 1 h before baseline assessment in a stable temperature and humidity room. Each rat was confined in clear plexiglas boxes placed on an elevated metal mesh floor. Von Frey filaments were applied perpendicularly to the central plantar surface of the ipsilateral hind paw for 5 s in ascending order of force (1.4 − 26 g). Paw withdrawal or licking was considered as a positive response and the next weaker filament was applied. In case of negative response (no paw withdrawal or licking), we then applied the next stronger filament. This paradigm continued until four measurements have been obtained after an initial change of behavior, or until four consecutive negative responses or five consecutive positive responses. The 50 % response threshold was calculated using the Up-Down method and Dixon's formulae (Dixon, 1980).

Thermal allodynia was assessed using the paw immersion test. One day before baseline assessment, rats were habituated to the test in order to get them used to the apparatus and the manual restraint. The ipsilateral hind paw to nerve ligation was immersed in a water bath maintained at 15.0 ± 0.2 °C until paw withdrawal or sign of struggle were observed and the paw withdrawal latency was measured (cut-off time, 15 s). A shortened duration of immersion indicated thermal allodynia. The percentage of the maximal possible effect (% MPE) was

Table 1
Detailed statistics for figures.

Figure #	Analysis	Statistics
Fig. 1A	2-way RM ANOVA	*F(24, 303)* = 8.261; P < 0.0001
Fig. 1B	2-way RM ANOVA	*F(18, 210)* = 8.647; P < 0.0001
Fig. 1C	1-way ANOVA	*F(15, 120)* = 3.917; P < 0.0001
Fig. 1D, 3E	1-way ANOVA	*F(4, 35)* = 6.621; P = 0.0004
Fig. 2A	2-way RM ANOVA	*F(8, 105)* = 0.3359; P = 0.9502
Fig. 2B	1-way ANOVA	*F(5, 25)* = 58.65; P < 0.0001
Fig. 2C	2-way RM ANOVA	*F(24, 261)* = 12.86; P < 0.0001
Fig. 2D	2-way RM ANOVA	*F(10, 126)* = 7.471; P < 0.0001
Fig. 2F	2-way RM ANOVA	*F(12, 136)* = 17.93; P < 0.0001
Fig. 2G	2-way RM ANOVA	*F(12, 136)* = 15.10; P < 0.0001
Fig. 2H	2-way RM ANOVA	*F(9, 80)* = 4.289; P = 0.0001
Fig. 3A, 3C	2-way RM ANOVA	*F(20, 210)* = 1.472; P = 0.0935
Fig. 3B, 3D	2-way RM ANOVA	*F(20, 180)* = 2.847; P = 0.0001
Fig. 3E, 1D	1-way ANOVA	*F(4, 35)* = 6.621; P = 0.0004
Fig. 4A	1-way ANOVA	*F(2, 17)* = 8.532; P = 0.027
Fig. 4B	1-way ANOVA	*F(2, 19)* = 13.47; P = 0.0002
Fig. 4C	1-way ANOVA	*F(3, 27)* = 10.08; P < 0.0001
Fig. 4D	1-way ANOVA	*F(2, 20)* = 5.319; P = 0.0141
Fig. 4E	1-way ANOVA	*F(4, 34)* = 4.486; P = 0.0051
Fig. 4F	1-way ANOVA	*F(4, 34)* = 12.24; P < 0.0001
Fig. 4G	1-way ANOVA	*F(3, 22)* = 4.64; P = 0.0116
Fig. 4H	2-way RM ANOVA	*F(3, 18)* = 4.937; P = 0.0113
Fig. 4I	2-way RM ANOVA	*F(3, 17)* = 5.628; P = 0.0072
Fig. 5A	1-tailed paired *t* test	P = 0.0446
Fig. 5B	1-way ANOVA	*F(4, 37)* = 4.299; P = 0.0059
Fig. 6A	2-way RM ANOVA	*F(28, 190)* = 10.84; P < 0.0001
Fig. 6B	2-way RM ANOVA	*F(24, 210)* = 7.252; P < 0.0001
Fig. 6C	1-wayANOVA	*F(4, 28)* = 7.078; P = 0.0005
Fig. 6D	1-way ANOVA	*F(4, 31)* = 4.017; P = 0.0097
Fig. 7A	2-way RM ANOVA	*F(24, 240)* = 9.379; P < 0.0001
Fig. 7B	1-way ANOVA	*F(4, 34)* = 8.527; P < 0.0001
Fig. 7C	2-way RM ANOVA	*F(10, 126)* = 2.124; P = 0.0271
Fig. 7D	2-way RM ANOVA	*F(10, 108)* = 4.537; P < 0.0001
Appendix Fig. 2A	1-way ANOVA	*F(4, 43)* = 11.66; P < 0.0001
Appendix Fig. 2B	1-way ANOVA	*F(4, 28)* = 22.12; P < 0.0001
Appendix Fig. 2C	1-way ANOVA	*F(3, 20)* = 5.2; P = 0.0081
Appendix Fig. 2D	1-way ANOVA	*F(4, 28)* = 36.3; P < 0.0001
Appendix Fig. 2E	1-way ANOVA	*F(4, 34)* = 10.97; P < 0.0001
Appendix Fig. 2F, 2 G	1-way ANOVA	*F(4, 35)* = 6.621: P = 0.0004
Appendix Fig. 2H	1-way ANOVA	*F(2, 21)* = 5.369; P = 0.0131
Appendix Fig. 2A, 2B, 2C, 2D, 2E, 2 F, 2 G, 2H	1-way ANOVA	*F(8, 59)* = 2.584; P = 0.0172
Appendix Fig. 4	1-way ANOVA	*F(5, 30)* = 8.044; P < 0.0001
Appendix Fig. 5A	1-way ANOVA	*F (7, 16)* = 1351; P < 0.0001
Appendix Fig. 5B	1-way ANOVA	*F (2, 8)* = 7527; P = 0.0145
Appendix Fig. 5C	1-way ANOVA	*F (3, 12)* = 1668; P = 0.0001
Appendix Fig. 5D	1-way ANOVA	*F (2, 16)* = 9271; P = 0.0021
Appendix Fig. 6A	2-way RM ANOVA	*F(12, 161)* = 18.8; P < 000.1
Appendix Fig. 6B	2-way RM ANOVA	*F(6, 72)* = 0.5653; P = 0.7565

calculated according to the formulae: % MPE = [(Post-drug latency – Pre-drug latency) / (15 – Pre-drug latency)] x 100.

Mechanical nociception to paw pressure was assessed using a Randall-Selitto analgesimeter (Ugo Basile, Bioseb, France) (Randall and Selitto, 1957) by applying increasing pressure to the left hind paw of the rat until a vocalization or a struggle response occurs, with a cut-off threshold of 750 g to avoid tissue damage.

The social recognition test (ability of an adult rat to recognize a younger conspecific during two 5-min sessions) was performed using a procedure without intersession delay (Loiseau et al., 2008). SNL and sham rats were individually housed for 2 days before testing. On the test day, under dim light conditions (30 lx), a juvenile rat was placed into the home cage for 2 consecutive 5-min sessions. The time spent in active social investigation (*i.e.* time spent by the adult rat in sniffing, following, biting, jumping, and crawling over or under the juvenile one) during the first (T_1) and the second (T_2) session was monitored. Social recognition was expressed as the difference between the exploration times during the two sessions (T_1-T_2) (Appendix Fig. 1A).

The novel object recognition (NOR) test provides a measure of episodic memory and was performed as previously described (Ennaceur and Delacour, 1988). Recognition memory is expressed as a recognition index, defined as the ratio of time spent exploring the novel object over the total time spent exploring both familiar and novel objects. One day before the test, SNL and sham rats were singly habituated to the test arena (75 × 75 × 50 cm) for 5 min. On the test day, rats were allowed to freely explore two objects (familiarization trial) under dim light conditions (30 lx) and returned to their home cage. After a 5-min inter-trial interval, rats were reintroduced in the arena with one of the original objects replaced by a novel one (recognition trial). The sequence of presentations and the location of the objects was randomly assigned to each rat. Both trials lasted 5 min. Exploration of each object was recorded separately. Exploratory behavior was defined as looking, licking, or touching the object while sniffing with active vibrissae. Novel object recognition memory was expressed as a recognition index (d2 score), defined as the ratio of time spent exploring the novel object over the total time spent exploring both familiar and novel objects (Appendix Fig. 1B).

In experiments using healthy rats, memory deficit was induced by intraperitoneal injection of phencyclidine (PCP, 5 mg/kg) 45 min before the familiarization trial. Then, the same procedure as for SNL rats was applied (Appendix Fig. 1C).

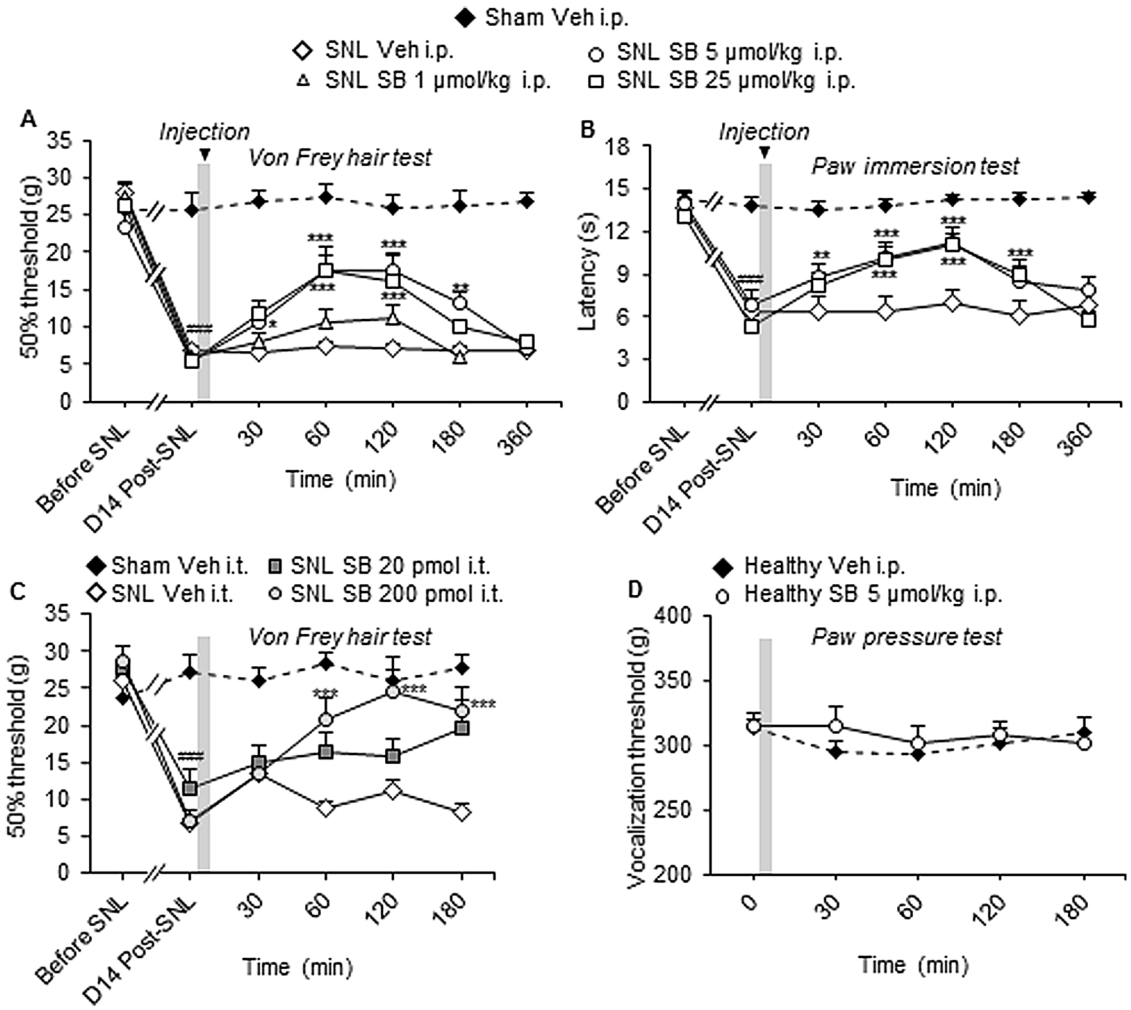


Fig. 1. Systemic and intrathecal administration of SB258585 reduces SNL-induced allodynia.
A, B. Intraperitoneal administration of SB258585 (SB, 1, 5 and 25 µmol/kg) but not vehicle (Veh, water for injections) improved tactile (**A**) and thermal (**B**) allodynia in SNL rats (n = 8/group). $^{###}P < 0.001$ *vs.* values measured before SNL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle (water) injection (D14 Post-SNL), 2-way ANOVA.
C. Intrathecal administration of SB258585 (SB, 200 pmol/rat) but not vehicle (Veh, water for injections) suppressed tactile allodynia in SNL rats (n = 6/group). $^{###}P < 0.001$ *vs.* values measured before SNL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle (water) injection (D14 Post-SNL), 2-way ANOVA.
D. Intraperitoneal administration of SB258585 (5 µmol/kg) or vehicle (water for injections) did not modify paw pressure-induced vocalization threshold in healthy rats (n = 8/group).

2.4. Intrathecal injections

Intrathecal (i.t.) injection was performed under isoflurane anesthesia (4% induction, 2% maintenance) according to a previously described procedure (Mestre et al., 1994). The anesthetized rats were held at the pelvic girdle and drug was delivered (10 µl volume) using a 25-gauge × 1-inch needle connected to a 50 µl Hamilton syringe inserted into the subarachnoidal space between lumbar vertebrae L5 and L6, eliciting a tail flick. The syringe was held in position for a few seconds after the injection.

2.5. Cell culture and transfection

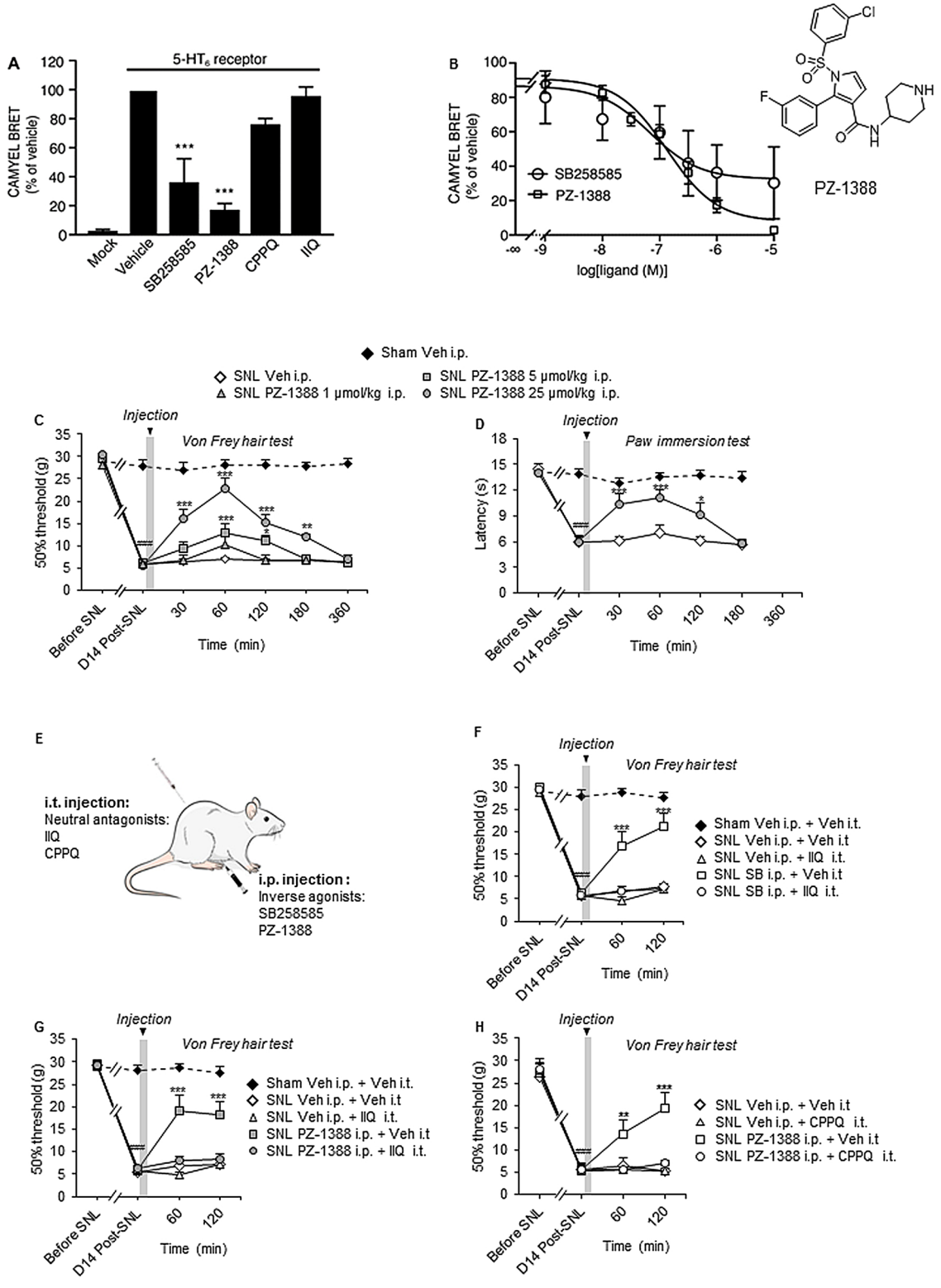
HEK-293 cells (ATCC) were cultured in DMEM (Gibco, Thermo Fisher Scientific) containing 10 % dialyzed fetal calf serum (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Sigma) at 37 °C in a 5% CO_2-enriched humidified atmosphere. Transfection (1 µg cDNA/well, 12-well plates) was performed with a Viromer ® RED kit (Lipocalyx), and the transfected cells were cultured in the same growth medium for 48 h before use. The pCMV-HA-5-HT_6, HA-5-HT_6-GFP, HA-5-HT_6 Δ^{391}, HA-5-HT_6 Δ^{413} plasmids were described elsewhere (Deraredj Nadim et al., 2016; Meffre et al., 2012) and the pCMV HA-5-HT_6 Δ^{426} was generated by directed mutagenesis (E^{426} in stop codon) using the QuickChange Site Directed Mutagenesis Kit (Agilent Technologies).

NG108 − 15 cells (ATCC HB12317) were grown in DMEM supplemented with 10 % dialyzed fetal calf serum, 2% hypoxanthine/aminopterin/thymidine (Life technologies), and antibiotics. They were co-transfected in suspension with 5-HT_6 receptor (0.5 µg DNA/million cells) and the Epac-based BRET sensor for cAMP (CAMYEL) (Jiang et al., 2007) constructs (1 µg DNA/million cells), using Lipofectamine 2000, according to the manufacturer's protocol. Transfected cells were plated in white 96-well plates (Greiner), at a density of 50,000 cells per well and used 24 h after transfection.

2.6. Western blot analysis

Cultured cells and tissues were sonicated, and solubilized in lysis buffer (75 mM Tris, 2 mM EDTA, 12 mM $MgCl_2$, 10 mM CHAPS, protease inhibitor mixture EDTA free (Roche cOmplete), pH 7.4) for 1 h at

4 °C. Samples were centrifuged at 12,000 × g for 20 min at 4 °C. Solubilized proteins were mixed with Laemmli buffer, heated at 70 °C for 10 min. Twenty-five µg protein were loaded for each lane, separated by 10 % acrylamide SDS-PAGE (TGX Stain-Free™ FastCast™, Biorad) (1.5 h at 120 V) in Tris-Glycine-SDS buffer and transferred to nitrocellulose membrane with a Trans-Blot® Turbo™ (Biorad) system. Membranes were blocked for 1 h in Tris-buffered saline containing 0.1 % Tween-20 and 5% BSA at room temperature and then incubated overnight at 4 °C with primary antibodies (anti phospho-Ser$^{240/244}$-S6, total S6, phospho-Ser^{2448}-mTOR, total mTOR and β-actin, all at 1:1,000 dilution, Cell Signaling Technologies). Membranes were further incubated with either anti-mouse or anti-rabbit HRP-conjugated secondary antibody (1:5,000 dilution, Thermo Scientific) and immunoreactivity was revealed using an enhanced chemiluminescence



(caption on next page)

Fig. 2. SB258585, PZ-1388, CPPQ and IIQ induce contrasting effects on receptor constitutive activity and SNL-induced allodynia.
A. Impact of SB258585, PZ-1388, CPPQ and IIQ (used at 10^{-6} M) on 5-HT_6 receptor constitutive activity (assessed by cAMP production measurement using the CAMYEL sensor) in NG108−15 cells. Data are the mean ± SEM of values obtained in at least three independent experiments performed on different sets of cultured cells. *** $P < 0.001$ *vs.* vehicle (ANOVA followed by Dunnett's test).
B. NG108−15 cells were exposed to incremental concentrations of either SB258585 or PZ-1388. Data are the mean ± SEM of triplicate determinations. Two independent experiments performed on different sets of cultured cells yielded similar results. Constitutive activity is represented as 100 % of the activity measured in the absence of compound (vehicle).
C, D. Intraperitoneal administration of PZ-1388 (1, 5 or 25 μmol/kg) but not vehicle (Veh, water for injections) improved tactile and thermal allodynia in SNL rats (n = 8/group). $^{###}P < 0.001$ *vs.* values measured before SNL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle (water) injection (D14 Post-SNL), 2-way ANOVA.
E. Schema illustrating the route of 5-HT_6 receptor ligand administrations in experiments illustrated on panels F–H.
F–H. Spinal 5-HT_6 receptor constitutive activity underlies tactile allodynia in SNL rats. Intrathecal administration of IIQ (2 nmol/rat) or CPPQ (2 nmol/rat) but not vehicle (Veh, water for injections) suppressed the anti-allodynic effect of SB258585 (SB, 5 μmol/kg, i.p.) and PZ-1388 (25 μmol/kg, i.p.) in SNL rats (n = 8/group). $^{###}P < 0.001$ *vs.* values measured before SNL; $^{**}P < 0.01$ $^{***}P < 0.001$ *vs.* values measured before drug/vehicle injection (D14 Post-SNL), 2-way ANOVA.

method (Clarity™ Western ECL, Biorad). Chemiluminescence signals were acquired using a high-resolution CCD camera (ChemiDoc™ XRS+, Biorad) and immunoreactive bands were quantified by densitometry using the Image Lab™ Biorad software.

2.7. Co-immunoprecipitation experiments

HEK-293 cells were transfected with HA-tagged mTOR and GFP-tagged 5-HT_6 constructs. Twenty-four hours after transfection, cells were washed in cold PBS, sonicated and solubilized in lysis buffer (75 mM Tris, 2 mM EDTA, 12 mM $MgCl_2$, 10 mM CHAPS, protease inhibitor cocktail EDTA free (Roche cOmplete), pH 7.4) for 5 h at 4 °C. Lysates were centrifuged at 12,000 x *g* for 20 min at 4 °C. Immunoprecipitations were performed using an anti-GFP antibody (Invitrogen, 1 μg per condition; overnight 4 °C incubation) and A/G plus agarose beads (Santa Cruz). Immunoprecipitated proteins and 50−100 μg of total proteins (lysates) were mixed with Laemmli buffer, heated at 70 °C for 10 min and separated by SDS-PAGE. Immunoblots were probed with anti-GFP (Roche) and anti-mTOR antibodies (1:1,000, Cell Signaling) and immunoreactivity was revealed using anti-rabbit, HRP-conjugated secondary antibodies. All Co-IP experiments were conducted from an identical amount of proteins (starting material); data were normalized over the quantity of immunoprecipitated 5-HT_6-GFP receptor and quantity of mTOR in the input.

2.8. Immunohistochemistry

Immunodetection of 5-HT_6 receptors was performed in allodynic male 5-HT_6-GFP KI mice. Animals were anesthetized with i.p. injection of 44 mg pentobarbital sodium (Ceva SA) and transcardially perfused with 4% (w/v) paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffered saline (PBS) fixative solution. The brain and the lumbar enlargement of the spinal cord were removed, post-fixed for 48 h in the same solution at 4 °C, before being cryoprotected with a 30 % (w/v) sucrose in 0.1 M PBS solution for 3 days at 4 °C. All tissues were embedded in tissue freezing medium (TFM, General Data Healthcare) and 40 μm-thick sections were cut at −27 °C with a cryotome (Microm KS34 & HM450, Thermo Scientific) and stored at 4 °C in 0.1 M PBS containing 0.1 % sodium azide. Sections were permeabilized and saturated with 0.2 % Triton X-100, 20 % goat serum in 0.1 M PBS for 1 h and incubated overnight at room temperature with the primary antibodies: anti-GFP (1:500, Invitrogen) and anti AC3 (1:1,000, Abcam) diluted in the same solution. Sections were then incubated for 1 h with Alexa Fluor 488 or 546-conjugated antibody (1:1,000, Sigma-Aldrich) in PBS containing 20 % goat serum. Immunofluorescence staining was observed with an Eclipse Ni-E microscope (Nikon) equipped with epifluorescence. Images were acquired using the NIS-Elements 4.13 software (Nikon) driving an ORCA Flash 4.0 camera (Hamamatsu).

2.9. Chemicals

SB258585 hydrochloride (5-HT_6 receptor antagonist, MW = 523.8 g.mol^{-1}), WAY181187 oxalate (5-HT_6 receptor agonist, MW = 470.9 g.mol^{-1}), phencyclidine hydrochloride (non-competitive NMDA receptor antagonist, MW = 279.9 g.mol^{-1}) and rapamycin (mTOR inhibitor, MW = 914.2 g.mol^{-1}) were purchased from Tocris Bioscience.

PZ-1388 hydrochloride (5-HT_6 receptor inverse agonist, 2-(3-fluorophenyl)-1-[(3-chlorophenyl)sulfonyl]-*N*-(piperidin-4-yl)-1H-pyrrole-3-carboxamide hydrochloride, MW = 498.4 g.mol^{-1}) was synthesized according to a multi-step procedure. Briefly, starting from methyl 2-(3-fluorophenyl)-1H-pyrrole-3-carboxylate obtained as previously reported (Grychowska et al., 2016), its acid derivative was coupled with 4-amino-1-Boc-piperidine providing *tert*-butyl 4-[2-(3-fluorophenyl)-1H-pyrrole-3-carboxamido]-piperidine-1-carboxylate. The final compound was obtained upon further sulfonylation, and removal of protecting group, to yield PZ-1388 as hydrochloride salt.

IIQ (5-HT_6 receptor neutral antagonist, 4-{[5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indol-1-yl]sulfonyl}isoquinoline dihydrochloride; MW = 492.42 g.mol^{-1}) and CPPQ (5-HT_6 receptor neutral antagonist, (*S*)-1-[(3-chlorophenyl)sulfonyl]-4-(pyrrolidine-3-yl-amino)-1H-pyrrolo[3,2-c]quinoline dihydrochloride; MW = 517.9 g.mol^{-1}), were synthesized as previously described (Grychowska et al., 2016; Zajdel et al., 2016).The Tat-VEPE ([NH2]YGRKKRRQRRR-FFVTDSVEPVE[COOH], purity >98 %, MW = 2711.1 g.mol^{-1}) and Tat-control ([NH2]YGRKKRRQRRR-TVNEK-VSCA[COOH], purity >98 %, MW = 2491.2 g.mol^{-1}) peptides were synthetized by Thermo Fisher Scientific. Oxaliplatin (antineoplastic agent, MW = 397.3 g.mol^{-1}) was purchased from Debiopharm.

2.10. Determination of cAMP production

NG108−15 cells transiently coexpressing the 5-HT_6 receptor and the CAMYEL probe were washed with PBS containing calcium and magnesium. Coelanterazine H (Molecular Probes) was added at a final concentration of 5 μM, and left at room temperature for 5 min. Cells were then treated with either vehicle or SB258585 or PZ-1388 or CPPQ or IIQ. BRET was measured using a Mithras LB 940 plate reader (Berthold Technologies). BRET values are expressed as percent of the activity measured in cells expressing the 5-HT_6 receptor, and treated with vehicle.

2.11. Statistical analyses

Data are expressed as means ± SEMs. The sample size for each experiment is expressed in each Figure legend. Biochemical and behavioral data were analyzed using a two-tailed Student's *t*-test, a 1-way ANOVA followed by Bonferroni's post-hoc test or a 2-way ANOVA followed by Dunnett's post-hoc test. The statistical significance was set at 5% ($P < 0.05$). Statistical analyses were run using GraphPad Prism 6.0 software. All statistics are detailed in Table 1.

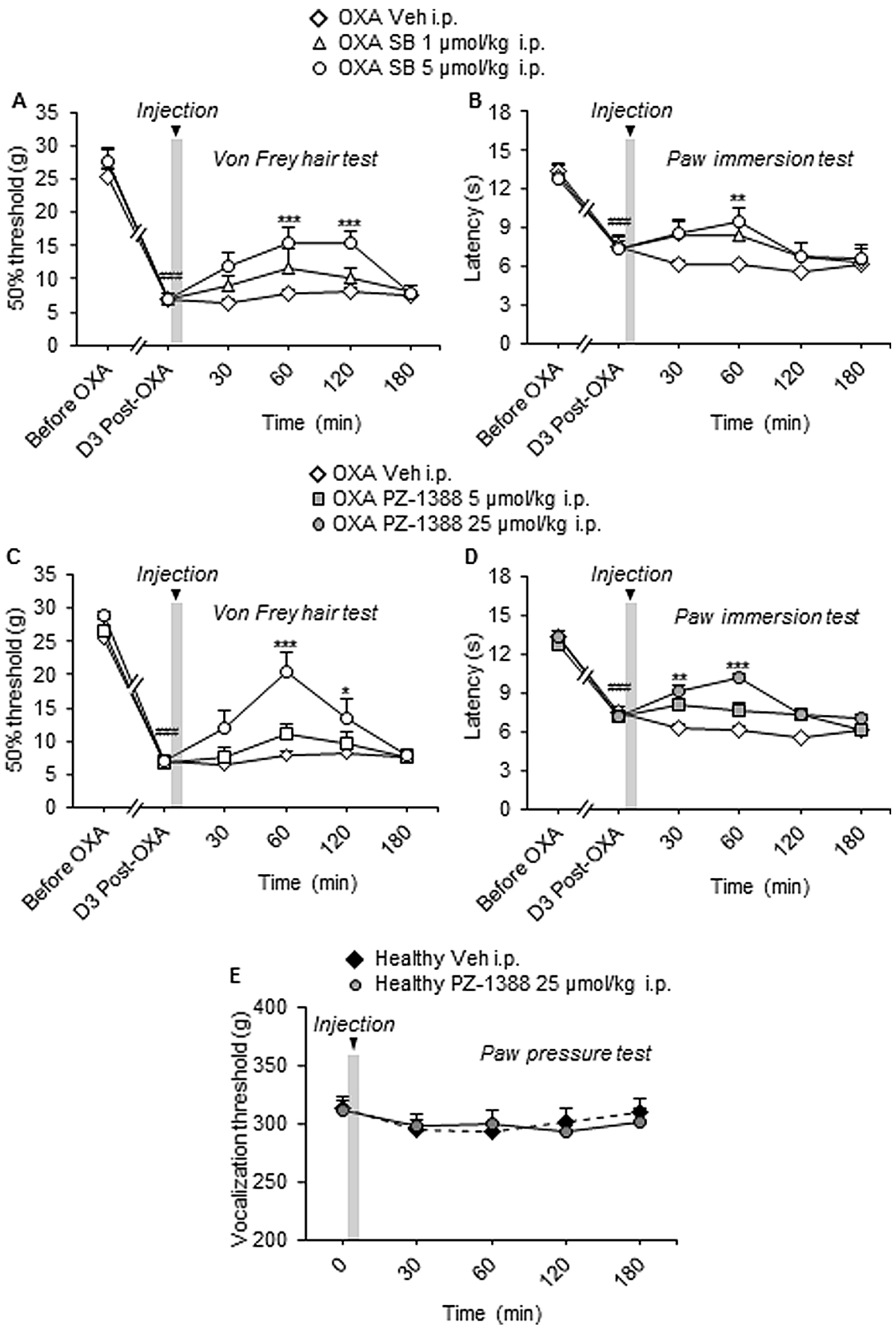


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Fig. 3. Systemic administration of SB258585 or PZ-1388 reduces OXA-induced tactile and thermal allodynia.
A, B. Intraperitoneal administration of SB258585 (SB, 1 and 5 μmol/kg) but not vehicle (Veh, water for injections) improved tactile and thermal allodynia in OXA rats (n = 8/group). $^{###}P < 0.001$ *vs.* values measured before SNL; $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle (water) injection (D3 Post-OXA), 2-way ANOVA.
C, D. Intrathecal administration of SB258585 (SB, 200 pmol/rat) but not vehicle (Veh, water for injections) suppressed tactile allodynia in SNL rats (n = 8/group). $^{###}P < 0.001$ *vs.* values measured before SNL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle (water) injection (D3 Post-OXA), 2-way ANOVA.
E. Intraperitoneal administration of PZ-1388 (25 μmol/kg) or vehicle (water for injection) did not modify paw pressure-induced vocalization threshold in healthy rats (n = 8/group).

3. Results

3.1. 5-HT_6 receptor blockade reduces tactile and thermal allodynia induced by spinal nerve ligation in the rat

As previously reported (Dupuis et al., 2017), 95 % of the SNL rats used in the present study displayed, 14 days after the surgery, tactile allodynia to plantar stimulation with von Frey monofilaments and thermal allodynia in response to a cold stimulus applied on the paw ipsilateral to the side of injury, as shown by the dramatic decrease in 50 % paw withdrawal thresholds (5.62 ± 0.14 *vs.* 28.30 ± 0.21 g before surgery, one-tailed paired *t* test $P < 0.0001$) and paw-withdrawal latencies (6.34 ± 0.29 *vs.* 13.70 ± 0.29 s before surgery, one-tailed paired *t* test $P < 0.0001$). The blockade of 5-HT_6 receptors with systemic administration of SB258585 (5 and 25 μmol/kg) reduced SNL-induced tactile allodynia 60–180 and 30 − 120 min after injection, respectively (Fig. 1A, Table 1). The maximal effect was similar for both doses (17.43 ± 2.09 g and 17.44 ± 3.12 g at 60 min, corresponding to 72.2 ± 9.5 % and 62.7 ± 20.2 % reversal, respectively). Both doses also produced a similar global anti-allodynic effect, as assessed by the area under the time-course curve (A.U.C.) of 50 % threshold variations (Appendix Fig. 2A, Table 1). Administration of SB258585 likewise increased paw-withdrawal latency to cold bath immersion (60–120 and 30 − 180 min after systemic injection of SNL rats with 5 and 25 μmol/kg, respectively (Fig. 1C, Table 1), indicating that 5-HT_6 receptor blockade also alleviates thermal allodynia. The maximal anti-allodynic effect, observed 120 min after injection of both doses, corresponded to 52.9 ± 9.0 % and 58.6 ± 1.5 % of maximal possible effect (MPE), respectively. As observed for tactile allodynia (see Fig. 1A), no gain of effect was obtained with the 25 μmol/kg dose, compared with 5 μmol/kg. Therefore, the 25 μmol/kg dose was discarded in further behavioral studies. In contrast, systemic administration of SB258585 (5 μmol/kg) failed to increase paw-pressure induced vocalization threshold in healthy rats (Fig. 1D, Table 1), indicating that 5-HT_6 receptor inhibition does not prevent mechanical nociception and specifically alleviates painful symptoms characteristic of neuropathic pain.

Reminiscent of the effects of its systemic administration, intrathecal injection of SB258585 in SNL rats (200 pmol/rat, i.t.) reversed tactile allodynia, as shown by the time-course (Fig. 1C, Table 1) and the A.U.C. of 50 % threshold variations (Appendix Fig. 2C, Table 1), indicative of a role of spinal 5-HT_6 receptors in SNL-induced allodynia. In light of these findings, we examined 5-HT_6 receptor expression and localization in the spinal cord. We used knock-in mice expressing a GFP-tagged version of the receptor (5-HT_6-GFP KI mice) (Deraredj Nadim et al., 2016) and visualized receptors with a GFP antibody. 5-HT_6 receptor immunostaining was mainly detected on cilia-like processes of dorsal horn spinal cord neurons of 5-HT_6-GFP KI mice (Appendix Fig. 3), consistent with previous observations made in several brain regions (Brodsky et al., 2017; Lesiak et al., 2018). Furthermore, 5-HT_6 receptors were co-localized with adenylyl cyclase 3 (AC3), an established marker of primary cilium (Appendix Fig. 3A). No 5-HT_6 receptor immunostaining was detected in dorsal root ganglia (Appendix Fig. 3B), consistent with previous observations (Hirst et al., 2003; Nicholson et al., 2003; Pierce et al., 1997).

3.2. Constitutively active spinal 5-HT_6 receptor mediates tactile and thermal allodynia in SNL rats

In line with previous findings indicating both high level of constitutive activity of 5-HT_6 receptors (Deraredj Nadim et al., 2016; Duhr et al., 2014; Grychowska et al., 2016; Kohen et al., 2001) and inverse agonist properties of SB258585 at 5-HT_6 receptor (Duhr et al., 2014), exposing NG108 − 15 cells expressing recombinant 5-HT_6 receptors to SB258585 strongly inhibited basal cAMP production elicited by receptor expression (Fig. 2A and B). This effect was reproduced by the newly developed arylsulfonamide of 2-aryl-1H-pyrrole-3-carboxamide derivative PZ-1388 (Fig. 2A and B). In contrast, the two potent and selective 5-HT_6 receptor antagonists, IIQ (Zajdel et al., 2016) and CPPQ (Deraredj Nadim et al., 2016; Grychowska et al., 2016) did not decrease basal cAMP level in NG108 − 15 cells (Fig. 2A, Table 1), indicating that they behave as neutral antagonists. Reminiscent of the SB258585 effects, and corroborating its inverse agonist effects on recombinant 5-HT_6 receptors, PZ-1388 administration to SNL rats dose-dependently reduced tactile allodynia (60–120 and 30 − 180 min after injection in rats treated with 5 and 25 μmol/kg of PZ-1388, respectively, Fig. 2C, Table 1). The maximal threshold elicited by the 5 and 25 μmol/kg doses (12.72 ± 2.26 g and 22.76 ± 2.49 g, respectively) was observed 60 min after injection and corresponded to a 26.8 ± 9.9 % and 68.4 ± 10.9 % reversal of allodynia, respectively (Fig. 2C). The global anti-allodynic effect of PZ-1388, assessed by the A.U.C. of 50 % threshold variations, confirmed a dose-dependent effect (Appendix Fig. 2B, Table 1). Therefore, the most efficient dose (25 μmol/kg) was further used in behavioral studies. Administration of PZ-1388 (25 μmol/kg) also increased paw-withdrawal latency to cold bath immersion 30 − 120 min after injection in SNL rats (Fig. 2D, Table 1). The maximal effect was observed 60 min after injection and resulted in 47.3 ± 14.1 % of MPE.

In contrast to the anti-allodynic effects of SB258585 and PZ-1388, the administration of the neutral antagonists IIQ or CPPQ did not modify the mechanical threshold to von Frey hair application (Fig. 2F-H, Table 1). This suggests that SNL-induced allodynia does not result from a tonic activation of spinal 5-HT_6 receptors elicited by endogenously released serotonin, but rather from receptor constitutive activity. To further support the role of constitutive activity of spinal 5-HT_6 receptor in neuropathic pain, we performed joint systemic administration of inverse agonists (SB258585 and PZ-1388) and intrathecal injection of neutral antagonists (Fig. 2E). Intrathecal administration of IIQ (2 nmol/rat, i.t.) totally prevented the anti-allodynic effects of systemic administration of SB258585 (Fig. 2F, Table 1) or PZ-1388 (Fig. 2G, Table 1) in SNL rats. Likewise, spinal injection (2 nmol/rat) of CPPQ suppressed the anti-allodynic effect induced by systemic administration of PZ-1388 (Fig. 2H, Table 1).

We next explored whether the anti-allodynic effects of SB258585 and PZ1388 observed in SNL-induced traumatic neuropathic pain could be extended to chemotherapy-induced peripheral neuropathy (CIPN), a major cause of chemotherapy dose reduction or cessation in patients with cancer (Balayssac et al., 2011; Seretny et al., 2014). In OXA-treated rats, systemic administration of SB258585 (5 μmol/kg) significantly improved tactile and thermal allodynia (Fig. 3A and B, Table 1). A.U.C. of paw withdrawal threshold variations confirmed the

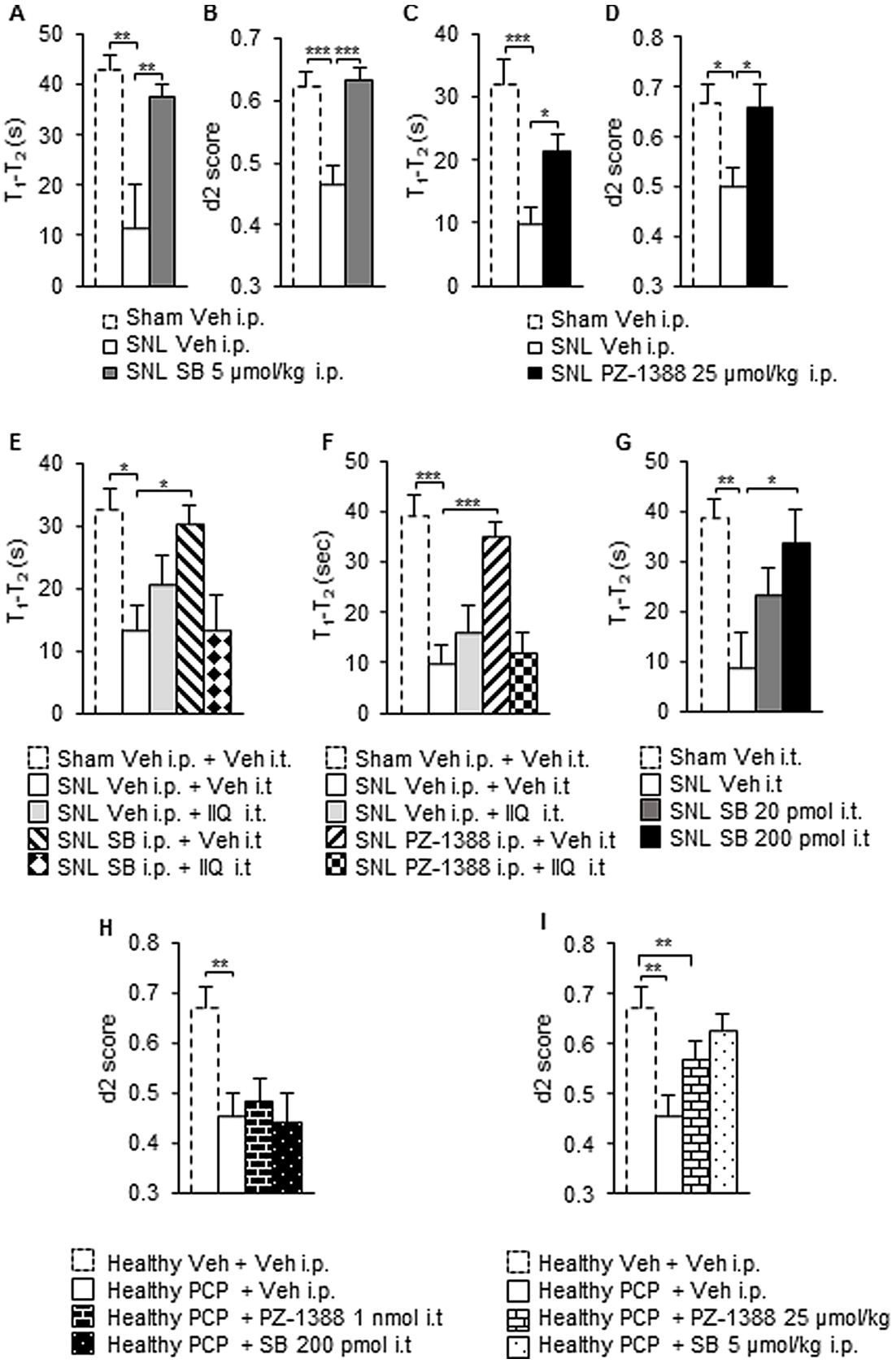

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Fig. 4. Effect of 5-HT$_6$ receptor inverse agonists on cognitive deficits induced by neuropathic pain or phencyclidine in rats.
A-D. Intraperitoneal administration of SB258585 (SB, 5 µmol/kg) or PZ-1388 (25 µmol/kg) but not vehicle (Veh, water for injections) restored social recognition and novelty discrimination in SNL rats (n = 8/group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 1-way ANOVA.
E, F. Intrathecal administration of IIQ (2 nmol/rat) but not vehicle (Veh, water for injections) suppressed the pro-cognitive effect of SB258585 (SB, 5 µmol/kg, i.p.) and PZ-1388 (25 µmol/kg, i.p.) in SNL rats (n = 8/group). The administration of IIQ with vehicle did not affect the social recognition deficit (n = 8/group). $^*P < 0.05$, $^{***}P < 0.001$, 1-way ANOVA.
G. Intrathecal administration of SB258595 (200 pmol/rat) but not vehicle (Veh, water for injections) restored social recognition performance in SNL rats (n = 8/group). $^*P < 0.05$, $^{**}P < 0.01$, 1-way ANOVA.
H, I. Intraperitoneal but not intrathecal administration of SB258585 (SB, 5 µmol/kg i.p. and 200 pmol/rat, i.t.) or PZ-1388 (25 µmol/kg, i.p. and 1 nmol/rat, i.t.) improved phencyclidine (PCP, 5 mg/kg, i.p.)-induced memory deficit in rats (n = 8/group). $^{**}P < 0.01$, 1-way ANOVA.

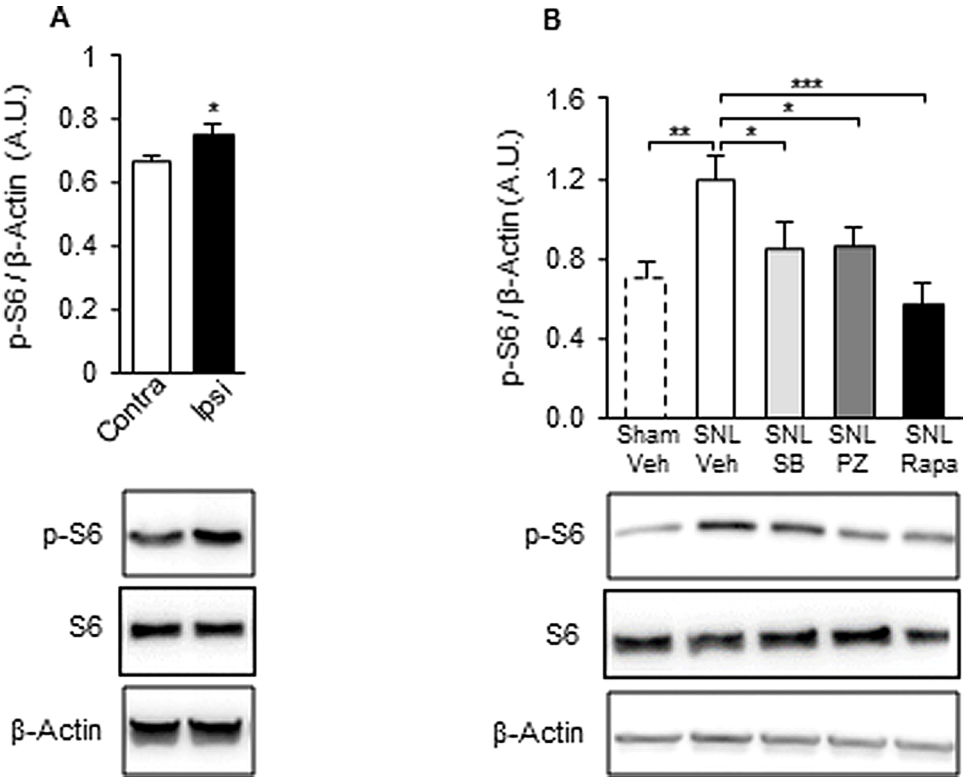

Fig. 5. 5-HT$_6$ receptor inverse agonists suppress SNL-induced increase of mTOR signaling in dorsal spinal cord.
A. SNL increased phosphorylation of S6 (at Ser$^{240/244}$) in the ispsilateral (Ipsi) side to injured nerve of the dorsal spinal cord, compared to contralateral (Contra) side (n = 3/group). $^*P < 0.05$, 1-tailed paired *t* test.
B. SNL increased phosphorylation of S6 (at Ser$^{240/244}$) in the ispsilateral (Ipsi) side to injured nerve of the dorsal spinal cord compared to Sham rats. SB258585 (SB, 5 µmol/kg, i.p.), PZ-1388 (PZ, 25 µmol/kg, i.p.) and rapamycin (Rapa, 10 nmol/rat, i.t.) suppressed SNL-induced increase in phospho-Ser$^{240/244}$ S6 (n = 7–11/group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 1-way ANOVA.

anti-allodynic effect of SB258585 (Appendix Fig. 2F, Table 1). Likewise, PZ-1388 administration significantly improved tactile and thermal allodynia in OXA-treated rats (Fig. 3C and D, Table 1 and Appendix Fig. 2G, Table 1). Finally, mimicking the effects of SB258585, PZ-1388 failed to increase paw-pressure induced vocalization threshold in healthy rats (Fig. 3E, Table 1).

Taken together, these data demonstrate that constitutively active spinal 5-HT$_6$ receptors contribute to neuropathic pain and, correspondingly, that 5-HT$_6$ receptor inverse agonists alleviate allodynic symptoms in both traumatic and chemically-induced neuropathy.

3.3. Blocking 5-HT$_6$ receptor constitutive activity improves co-morbid cognitive symptoms associated with painful neuropathy in SNL rats

In line with the pro-cognitive effects of 5-HT$_6$ receptor blockade in several rodent models of cognitive impairment (Gravius et al., 2011; Hirst et al., 2006; Loiseau et al., 2008; Vanda et al., 2018; Zajdel et al., 2016), we next examined whether inhibiting 5-HT$_6$ receptor constitutive activity would also improve cognitive deficits associated with chronic neuropathic pain in SNL rats. We first evaluated the influence of SB258585 and PZ-1388 upon social cognition (social interaction test) and episodic memory (NOR test). SNL-allodynic rats treated with vehicle failed to recognize a younger conspecific rat compared to sham rats, and to discriminate a novel object from the familiar one (Fig. 4A-G, Table 1). Administration of SB258585 (5 µmol/kg, i.p..) totally restored novelty discrimination in the social recognition (Fig. 4A, Table 1) and NOR (Fig. 4B, Table 1) tests in SNL rats. Injection of PZ-1388 (25 µmol/kg, i.p.) attenuated the social recognition deficit (Fig. 4C) and abolished the episodic memory deficit elicited by SNL (Fig. 4D, Table 1). Importantly, no signs of abnormal behavior were detected in the allodynic SNL rats due to the systemic administration of either SB258585 or PZ-1388. Altogether, these data show pro-cognitive effects of systemic administration of 5-HT$_6$ receptor inverse agonists to painful neuropathic rats.

To further explore whether the pro-cognitive effects of SB258585 and PZ-1388 in SNL rats result from neuropathic pain improvement mediated by constitutively active spinal 5-HT$_6$ receptors, we intrathecally injected the neutral antagonist IIQ concomitantly with the systemic administration of inverse agonists to specifically inhibit the inverse agonist effects in the spinal cord (Appendix Fig. 1A). Intrathecal administration of IIQ (2 nmol/rat, i.t.), which abolished the anti-allodynic effect of SB258585 (5 µmol/kg) and PZ-1388 (25 µmol/kg, see Fig. 2F and G, Table 1), also suppressed the pro-cognitive effect of both inverse agonists (Fig. 4E and F, Table 1). Furthermore, the intrathecal injection of SB258585 in SNL rats (200 pmol/rat, i.t.) restored mnemonic performance in the social interaction test (Fig. 4G, Table 1), thereby reproducing the effects of a systemic administration.

To determine whether the pro-cognitive effects of SB258585 and PZ-1388 observed in SNL rats result from neuropathic pain improvement, we examined their effect in a model of cognitive deficit induced

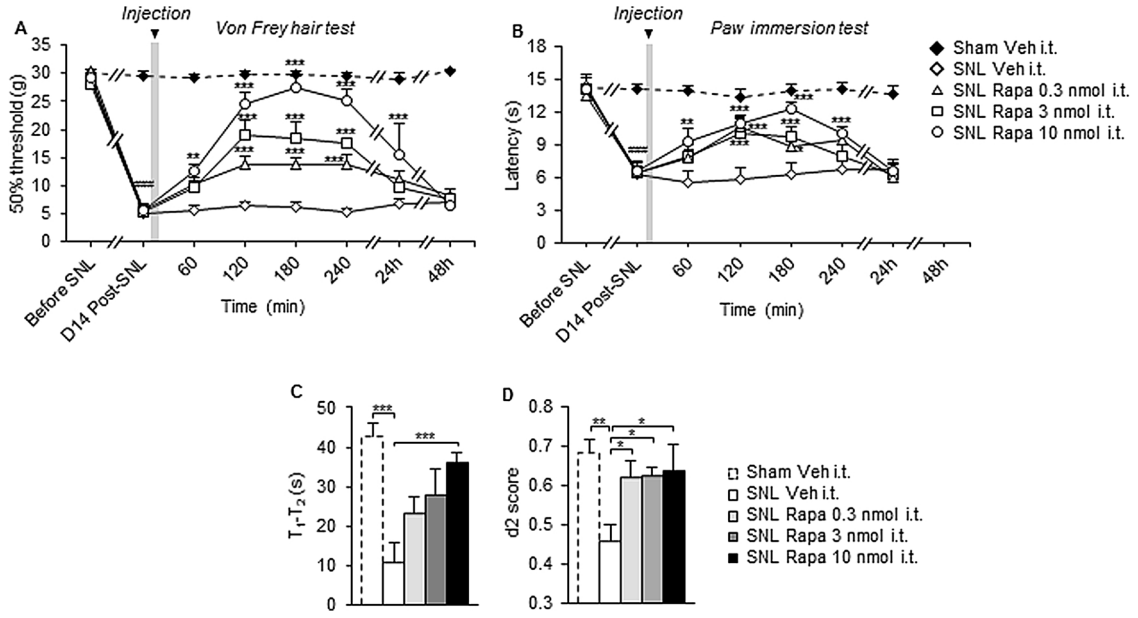

Fig. 6. Rapamycin administration alleviates tactile and thermal allodynia and cognitive co-morbidities in SNL rats.
A, **B**. Intrathecal administration of rapamycin (Rapa, 0.3 − 10 nmol/rat) but not vehicle (Veh, water for injections) dose-dependently increased the 50 % threshold to von Frey hair application and the response latency to paw immersion in cold water (15 °C) in SNL rats (n = 6–7/group). $^{###}P < 0.001$ *vs.* values measured before SNL. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle injection (D14 Post-SNL), 2-way ANOVA.
C, **D**. Intrathecal administration of rapamycin (Rapa, 0.3 − 10 nmol/rat) but not vehicle (Veh, water for injections) restored social and novel object recognition performance in SNL rats(n = 8/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 1-way ANOVA.

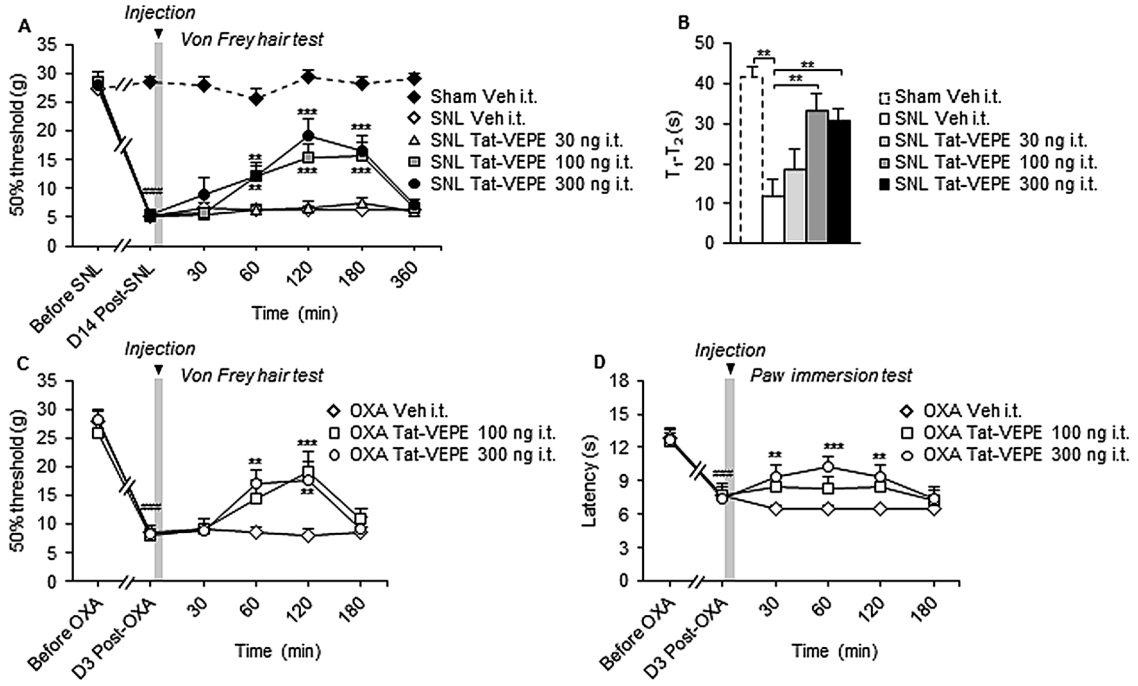

Fig. 7. Intrathecal administration of Tat-VEPE reduces SNL-induced tactile allodynia, the associated social recognition deficits and OXA-induced tactile and thermal allodynia.
A. Intrathecal administration of Tat-VEPE (100 and 300 ng/rat) but not vehicle (Veh, water for injections) increased the 50 % threshold to von Frey hair application in SNL rats (n = 7-8/group). $^{###}P < 0.001$ *vs.* values measured before SNL; $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle injection (D14 Post-SNL), 2-way ANOVA.
B. Tat-VEPE (100 and 300 ng/rat) but not vehicle (Veh, water for injections) restored social recognition performance in SNL rats (n = 8/group). $^{**}P < 0.01$, 1-way ANOVA.
C, **D**. Effect of intrathecal administration of Tat-VEPE (100 and 300 ng/rat) on the 50 % paw withdrawal threshold to von Frey hair application (**C**) and the response latency to paw immersion in cold water (15 °C) (**D**) in OXA rats (n = 8/group). $^{###}P < 0.001$ *vs.* values measured before OXA; $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* values measured before the drug/vehicle injection (D3 Post-OXA), 2-way ANOVA.

by i.p. injection of phencyclidine (PCP) in healthy (non-neuropathic) rats (Appendix Fig. 1C). Intrathecal administration of SB258585 (200 pmol/rat) or PZ-1388 (1 nmol/rat) did not restore novelty discrimination in the NOR test (Fig. 4H) in non-neuropathic rats treated with PCP, whereas systemic administration of SB258585 (5 μmol/kg) and PZ-1388 (25 μmol/kg) abolished PCP-induced novelty discrimination deficit (d2 index: 0.634 ± 0.062 and 0.567 ± 0.040, respectively, no significant difference in comparison with control rats, Fig. 4I). Collectively, these results indicate that the pro-cognitive effects of SB258585 and PZ-1388 in SNL rats depend on their anti-allodynic effect mediated by the blockade of constitutively active spinal 5-HT_6 receptors.

3.4. Agonist-independent 5-HT_6 receptor-operated mTOR signaling underlies neuropathic pain and associated cognitive dysfunction in SNL rats

As 5-HT_6 receptors are known to engage mTOR signaling (Meffre et al., 2012; Teng et al., 2019; Wang et al., 2015) and mTOR has been implicated in neuropathic pain (Duan et al., 2018; Geranton et al., 2009; He et al., 2019; Obara et al., 2011; Wang et al., 2016; Zhang et al., 2013), we examined whether SNL affects mTOR activity in dorsal horn of lumbar spinal cord. Phosphorylation of ribosomal S6 protein at $Ser^{240/244}$, a downstream substrate of mTOR, was significantly increased in the ipsilateral side to the nerve ligation, compared with the contralateral side (Fig. 5A, Table 1). As expected, spinal administration of the mTOR inhibitor rapamycin (10 nmol/rat i.t.) to SNL rats abolished SNL-induced elevation of $Ser^{240/244}$ phosphorylation (Fig. 5B, Table 1). We thus reasoned that the enhanced mTOR activation in the ipsilateral spinal cord dorsal horn of SNL rats would result from the constitutive activity of spinal 5-HT_6 receptors. We first demonstrated that the basal level of mTOR phosphorylation (at Ser^{2448}, measured as an index of mTOR activation state) in HEK-293 cells expressing 5-HT_6 receptor was significantly reduced by SB258585 or PZ-1388, but not IIQ exposure (Appendix Fig. 4, Table 1). This indicates that 5-HT_6 receptors constitutively activate mTOR signaling and that SB258585 and PZ-1388 behave as inverse agonists while IIQ behaves as neutral antagonist with respect of receptor-operated mTOR activation, corroborating their pharmacological properties on cAMP production (see Fig. 2A and B). Correspondingly, intrathecal injection of SB258585 or PZ-1388 significantly reduced the level of phosphorylated S6 in the dorsal spinal cord of SNL rats (Fig. 5B, Table 1), suggesting that the enhanced mTOR activity measured in these rats results, at least in part, from agonist-independent activation of spinal 5-HT_6 receptors.

We then examined whether mTOR activation underlies tactile and thermal allodynia in SNL rats. Reminiscent of the effects of 5-HT_6 receptor inverse agonists, intrathecal administration of rapamycin (0.3, 3 and 10 nmol/rat) increased mechanical thresholds to von Frey hair application, with a maximal effect measured at 120 − 240 min post-injection (Fig. 6A, Table 1). The anti-allodynic effect of rapamycin 10 nmol was still significant 24 h after injection but disappeared at 48 h post-injection (Fig. 6A, Table 1). The global anti-allodynic effect of rapamycin, assessed by the A.U.C. of 50 % threshold variations, showed a dose-dependent effect (Appendix Fig. 2D, Table 1). Rapamycin treatment also increased paw-withdrawal latency to cold immersion, with a maximal effect observed 180 min after injection, but this anti-allodynic effect persisted no more than 240 min (Fig. 6B, Table 1). In line with its effects upon tactile and thermal allodynia, rapamycin dose-dependently reduced SNL-induced social recognition memory deficit (Fig. 6C, Table 1) and restored normal novel object recognition in SNL rats (Fig. 6D, Table 1).

3.5. Disrupting 5-HT_6 receptor-mTOR interaction improves neuropathic pain symptoms in SNL rats

We previously demonstrated that mTOR physically associates with the 5-HT_6 receptor C-terminal (Ct) domain and that this interaction is essential for receptor-mediated mTOR activation (Meffre et al., 2012). Expression of several Ct truncation mutants of the 5-HT_6 receptor in HEK-293 cells showed that the 21 Ct residues of the receptor are necessary to the engagement of mTOR signaling upon agonist stimulation of 5-HT_6 receptor (Appendix Fig. 5A). Among these residues, we identified a sequence of 10 amino acids (FFVTDSVEPE) showing homology with the mTOR recognition motif in the S6 protein. We then generated a cell-permeable interfering peptide that encompasses this 10 amino acid sequence fused to the transduction domain (YGRKKRRQ-RRR) of the HIV Tat protein (Tat-VEPE). Treatment of HEK-293 cells with the Tat-VEPE peptide strongly reduced 5-HT_6 receptor-mTOR interaction (Appendix Fig. 5B, Table 1) as well as agonist-dependent (Appendix Fig. 5C, Table 1) and independent (Appendix Fig. 5D, Table 1) mTOR activation.

Intrathecal administration of Tat-VEPE (100 and 300 ng/rat i.t., Fig. 7A) but not a control peptide (Tat-cont, Appendix Fig. 6A), reduced tactile allodynia for 180 min (Table 1). The maximal anti-allodynic effect occurred at 120 min post-injection, resulting in a 60.4 ± 13.7 % reversal of tactile allodynia. The A.U.C. of threshold variations confirmed the effect of both 100 and 300 ng/rat doses (Appendix Fig. 2E, Table 1). Reminiscent of the pro-cognitive effects of 5-HT_6 receptor inverse agonists and rapamycin, injection of Tat-VEPE also abolished social memory deficit in SNL rats (Fig. 7B, Table 1). The Tat-VEPE peptide also significantly increased paw withdrawal thresholds upon stimulation with von Frey monofilament in OXA rats, resulting in a 49.6 ± 14.9 % of reversal of tactile allodynia at 120 min for the 300 ng/rat dose (Fig. 7C and Appendix Fig. 2H, Table 1), as well as the reaction latency to a cold stimulus, the maximal effect resulting in 36.3 ± 6.3 % of reversal of thermal allodynia (Fig. 7D, Table 1). These anti-allodynic effects lasted 120 min *i.e.* a shorter duration than that observed in SNL rats (180 min). Corroborating the lack of antinociceptive effects of SB258585 and PZ-1388 in healthy rats, intrathecal injection of Tat-VEPE did not change paw pressure-induced vocalization threshold in non-neuropathic rats (Appendix Fig. 6B, Table 1).

4. Discussion

Active conformations of GPCRs are not only promoted by agonists, but can also occur in their absence, leading to constitutive activity (De Deurwaerdère et al., 2020) Constitutive activity has been established for numerous GPCRs in transfected cells expressing high receptor densities, but often remains to be demonstrated for native receptors. The 5-HT_6 receptor is a GPCR exhibiting a high level of constitutive activity both in recombinant cells and *in vivo* (Chaumont-Dubel et al., 2019; Deraredj Nadim et al., 2016; Duhr et al., 2014; Kohen et al., 2001). Here, we provide a convergent set of results indicating that 5-HT_6 receptor constitutive activity is involved in painful symptoms in two preclinical models of traumatic (SNL) and toxic (OXA administration) neuropathy of high translational value. In both models, systemic administration of the 5-HT_6 receptor inverse agonists SB258585 and PZ-1388 (one of the leads from the newly designed arylpyrrole carboxamide series), attenuated tactile and thermal allodynia associated with traumatic nerve injury and chemotherapy, while 5-HT_6 receptor ligands that behave as neutral antagonists were ineffective. The anti-allodynic effects of 5-HT_6 receptor inverse agonists were of similar magnitude in both models, emphasizing the relevance of blocking 5-HT_6 receptor constitutive activity for the management of neuropathic pains of different etiologies. Interestingly, these anti-allodynic effects were long-lasting, as usually observed with gabapentin, one of the reference treatments in neuropathic pain (Hunter et al., 1997; Pastrana-Quintos et al., 2020). Moreover, they were observed at much lower doses than the gabapentin doses (100 mg/kg p.os or 300 mg/kg, i.p.) necessary to produce a similar anti-allodynic effect (50 % reversal of pain) (Hunter et al., 1997; Pastrana-Quintos et al., 2020), minimizing the risk of off-target adverse effects.

The anti-allodynic effects of SB258585 in neuropathic rats

corroborates previous studies indicating that this compound improves chemical hyperalgesia (Castaneda-Corral et al., 2009; Godinez-Chaparro et al., 2012), tactile allodynia in SNL rats (Pineda-Farias et al., 2017) and thermal hyperalgesia in STZ-induced diabetic mice (Sari et al., 2019). The present study suggests that these previously described effects result from SB258585 inverse agonist activity at 5-HT_6 receptors. Further supporting that SNL-induced allodynia involves 5-HT_6 receptor constitutive activity rather than tonic activation of spinal 5-HT_6 receptors elicited by endogenously released serotonin, intrathecal administration of a neutral antagonist prevented the antiallodynic effects of SB258585 and PZ-1388. These findings together with the antiallodynic effect induced by intrathecal injection of SB258585 demonstrate a crucial role of constitutively active 5-HT_6 receptors located in the spinal cord.

Corroborating these observations and previous findings that revealed the presence of 5-HT_6 receptor mRNA in the dorsal horn spinal cord (Ward and Dorsa, 1996), we provide direct evidence of 5-HT_6 receptor expression in dorsal spinal cord neurons of 5-HT_6-GFP KI mice. The present results are also consistent with recent observations made in BAC-CreER mouse line where the 5-HT_6 receptor was found in excitatory interneurons of the dorsal spinal cord in a region below lamina II inner to lamina IV outer called “low-threshold mechanoreceptor-recipient zone” and involved in tactile perception processing (Abraira et al., 2017). Furthermore, we show that 5-HT_6 receptors are mainly localized in the primary cilium of dorsal spinal cord neurons, corroborating previous observations in striatal neurons where 5-HT_6 receptors were found to finely regulate cilia length and signaling as well as dendrite outgrowth and neuronal morphology (Brodsky et al., 2017; Lesiak et al., 2018). The functional relevance of the specific location of 5-HT_6 receptors in primary cilium of spinal cord neurons remains to be established.

Beyond the demonstration that spinal 5-HT_6 receptors are constitutively active in experimental models of neuropathic pain and that their blockade with inverse agonists alleviates allodynic symptoms, we show for the first time that 5-HT_6 receptors constitutively activate a non-canonical signaling pathway (mTOR signaling), as assessed by measuring the phosphorylation of mTOR (at Ser^{2448}) and its downstream substrate S6 (at $Ser^{240/244}$). To our knowledge, these findings provide the first demonstration of agonist-independent activation of mTOR signaling by a GPCR. Constitutive activation of mTOR was prevented by an interfering peptide disrupting 5-HT_6 receptor/mTOR physical interaction, suggesting that this interaction might stabilize the receptor in an active conformation able to stimulate mTOR kinase in an agonist-independent manner. Importantly, we established constitutive activity of 5-HT_6 receptor at mTOR signaling not only in HEK-293 cells transiently expressing recombinant receptors, but also in the spinal cord dorsal horn of SNL rats, specifically in the ipsilateral side of the lesion.

Furthermore, mimicking the effects of 5-HT_6 receptor inverse agonists, intrathecal delivery of the Tat-VEPE peptide reduced tactile allodynia with the same magnitude in SNL and OXA rats. These results indicate that the activation of mTOR, under the control of constitutively active spinal 5-HT_6 receptors, might contribute to tactile and thermal allodynia induced by traumatic nerve injury and chemotherapy. Similarly, intrathecal administration of rapamycin reversed SNL-induced neuropathic pain. The global effect of rapamycin, measured by the A.U.C. was similar to that of SB258585, PZ-1388 and Tat-VEPE. This antiallodynic effect was long-lasting (between 4 and 24 h) and more prolonged than that of inverse agonists, suggesting the involvement of additional (5-HT_6 receptor-independent) mechanisms, such as neurotrophin-induced activation of Trk receptors and mTOR signaling (Pezet, 2014). These results are also consistent with recent findings showing that i) mTOR signaling plays a central role in the sensitization of DRG and dorsal horn spinal cord neurons (Uttam et al., 2018) contributing to the development of chronic pain, and ii) intrathecal or systemic administration of rapalogs alleviates painful symptoms in various rodent models of neuropathic pain of different etiologies (Asante et al., 2010; Duan et al., 2018; He et al., 2019; Obara et al., 2011; Wang et al., 2016; Zhang et al., 2013).

The present study suggests that 5-HT_6 receptor inverse agonists currently in clinical development as symptomatic treatment of cognitive impairment in dementia and psychoses, might also be proposed for alleviating neuropathic pains. 5-HT_6 receptor inverse agonists are generally well tolerated (Atri et al., 2018) and thus certainly more relevant than mTOR inhibitors which exhibit strong adverse effects linked to their immunosuppressive activity. Disrupting the 5-HT_6 receptor/mTOR interaction might also be considered, as strategies based on interfering peptides to disrupt membrane receptor interaction with intracellular proteins (DOP receptor/Cdk5 (Beaudry et al., 2015); 5-HT_{2A} receptor/PSD-95 (Pichon et al., 2010); NMDA receptor/Src (Liu et al., 2008)) have already proved their efficacy in animal models of chronic pain.

Notably, blocking 5-HT_6 receptor-mediated mTOR signaling not only improved painful symptoms but also co-morbid cognitive deficits in neuropathic rats. Again, only the 5-HT_6 receptor inverse agonists SB258585 and PZ-1388 restored normal social and episodic memory in SNL rats, whereas a neutral antagonist (IIQ) failed to reverse cognitive deficits, underscoring the need for developing 5-HT_6 receptor inverse agonists rather than neutral antagonists for the treatment of neuropathic pain and co-morbid symptoms. The pro-cognitive effects of 5-HT_6 receptor antagonists (or inverse agonists) in various models of cognitive impairment such as scopolamine or PCP administration are mediated by the blockade of striatal, hippocampal and prefrontal 5-HT_6 receptors and rely on their abilities to restore normal cholinergic, GABAergic and glutamatergic transmissions in these brain regions (Codony et al., 2011). In contrast, the present results suggest that the beneficial effects of 5-HT_6 receptor inverse agonists upon cognitive impairment caused by neuropathic pain are independent of a supraspinal (cortico-limbic) mechanism but rely on the spinal analgesic effect of the compounds.

5. Conclusions

The present findings identify the activation of mTOR signaling by constitutively active spinal 5-HT_6 receptors as a key mechanism underlying tactile and thermal allodynia elicited by SNL or OXA administration in the rat. They provide the first demonstration of a pathological influence of agonist-independent activation of non-canonical signaling by a GPCR. They suggest that targeting constitutively active 5-HT_6 receptors or the 5-HT_6 receptor/mTOR complex might be efficient strategies to alleviate painful symptoms and associated cognitive comorbidities in neuropathic pain.

Acknowledgments

The authors wish to thank Prof Joël Bockaert for critical reading of the manuscript and his valuable suggestions. This work was supported by the Agence Nationale de la Recherche (ANR) (N° ANR-17-CE16-0010-01, ANR-17-CE16-0013-01, ANR-19-CE18-0018 and program “Investissements d’Avenir”16-IDEX-0001 CAP 20-25), the Institut National de la Santé et de la Recherche Médicale (INSERM), The Centre National de la Recherche Scientifique (CNRS), the Auvergne-Rhône-Alpes Region (France), the Université Clermont Auvergne, the Université de Montpellier and the National Science Centre, Poland (grant N° 2016/21/B/NZ7/01742).

Appendix A. The Peer Review Overview and Supplementary data

The Peer Review Overview and Supplementary data associated with this article can be found in the online version: https://doi.org/10.1016/j.pneurobio.2020.101787.

References

Abraira, V.E., Kuehn, E.D., Chirila, A.M., Springel, M.W., Toliver, A.A., Zimmerman, A.L., Orefice, L.L., Boyle, K.A., Bai, L., Song, B.J., Bashista, K.A., O'Neill, T.G., Zhuo, J., Tsan, C., Hoynoski, J., Rutlin, M., Kus, L., Niederkofler, V., Watanabe, M., Dymecki, S.M., Nelson, S.B., Heintz, N., Hughes, D.I., Ginty, D.D., 2017. The cellular and synaptic architecture of the mechanosensory dorsal horn. Cell 168, 295–310. https://doi.org/10.1016/j.cell.2016.12.010. e19.

Asante, C.O., Wallace, V.C., Dickenson, A.H., 2010. Mammalian target of rapamycin signaling in the spinal cord is required for neuronal plasticity and behavioral hypersensitivity associated with neuropathy in the rat. J. Pain 11, 1356–1367. https://doi.org/10.1016/j.jpain.2010.03.013.

Atri, A., Frölich, L., Ballard, C., Tariot, P.N., Molinuevo, J.L., Boneva, N., Windfeld, K., Raket, L.L., Cummings, J.L., 2018. Effect of Idalopirdine as adjunct to cholinesterase inhibitors on change in cognition in patients with Alzheimer disease: three randomized clinical trials. JAMA 319, 130. https://doi.org/10.1001/jama.2017.20373.

Balayssac, D., Ferrier, J., Descoeur, J., Ling, B., Pezet, D., Eschalier, A., Authier, N., 2011. Chemotherapy-induced peripheral neuropathies: from clinical relevance to preclinical evidence. Expert Opin. Drug Saf. 10, 407–417. https://doi.org/10.1517/14740338.2011.543417.

Beaudry, H., Mercier-Blais, A.-A., Delaygue, C., Lavoie, C., Parent, J.-L., Neugebauer, W., Gendron, L., 2015. Regulation of μ and δ opioid receptor functions: involvement of cyclin-dependent kinase 5: CDK5 regulates MOP and DOP receptor activity. Br. J. Pharmacol. 172, 2573–2587. https://doi.org/10.1111/bph.13088.

Bouhassira, D., Lantéri-Minet, M., Attal, N., Laurent, B., Touboul, C., 2008. Prevalence of chronic pain with neuropathic characteristics in the general population. Pain 136, 380–387. https://doi.org/10.1016/j.pain.2007.08.013.

Brodsky, M., Lesiak, A.J., Croicu, A., Cohenca, N., Sullivan, J.M., Neumaier, J.F., 2017. 5-HT6 receptor blockade regulates primary cilia morphology in striatal neurons. Brain Res. 1660, 10–19. https://doi.org/10.1016/j.brainres.2017.01.010.

Castaneda-Corral, G., Rocha-Gonzalez, H.I., Araiza-Saldana, C.I., Ambriz-Tututi, M., Vidal-Cantu, G.C., Granados-Soto, V., 2009. Role of peripheral and spinal 5-HT6 receptors according to the rat formalin test. Neuroscience 162, 444–452. https://doi.org/10.1016/j.neuroscience.2009.04.072.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.

Chaumont-Dubel, S., Dupuy, V., Bockaert, J., Bécamel, C., Marin, P., 2019. The 5-HT6 receptor interactome: new insight in receptor signaling and its impact on brain physiology and pathologies. Neuropharmacology 107839. https://doi.org/10.1016/j.neuropharm.2019.107839.

Chenaf, C., Delorme, J., Delage, N., Ardid, D., Eschalier, A., Authier, N., 2018. Prevalence of chronic pain with or without neuropathic characteristics in France using the capture-recapture method: a population-based study. Pain 159, 2394–2402. https://doi.org/10.1097/j.pain.0000000000001347.

Codony, X., Vela, J.M., Ramírez, M.J., 2011. 5-HT(6) receptor and cognition. Curr. Opin. Pharmacol. 11, 94–100. https://doi.org/10.1016/j.coph.2011.01.004.

De Deurwaerdère, P., Bharatiya, R., Chagraoui, A., Di Giovanni, G., 2020. Constitutive activity of 5-HT receptors: factual analysis. Neuropharmacology 168, 107967. https://doi.org/10.1016/j.neuropharm.2020.107967.

Deraredj Nadim, W., Chaumont-Dubel, S., Madouri, F., Cobret, L., De Tauzia, M.-L., Zajdel, P., Bénédetti, H., Marin, P., Morisset-Lopez, S., 2016. Physical interaction between neurofibromin and serotonin 5-HT $_6$ receptor promotes receptor constitutive activity. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.1600914113. 201600914.

Devegowda, V.N., Hong, J.R., Cho, S., Lim, E.J., Choo, H., Keum, G., Rhim, H., Nam, G., 2013. Synthesis and the 5-HT6 receptor antagonistic effect of 3-arylsulfonylamino-5,6-dihydro-6-substituted pyrazolo[3,4]pyridinones for neuropathic pain treatment. Bioorg. Med. Chem. Lett. 23, 4696–4700. https://doi.org/10.1016/j.bmcl.2013.05.100.

Dixon, W.J., 1980. Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. 20, 441–462. https://doi.org/10.1146/annurev.pa.20.040180.002301.

Duan, Z., Li, J., Pang, X., Wang, H., Su, Z., 2018. Blocking mammalian target of rapamycin (mTOR) alleviates neuropathic pain induced by chemotherapeutic bortezomib. Cell. Physiol. Biochem. 48, 54–62. https://doi.org/10.1159/000491662.

Duhr, F., Déléris, P., Raynaud, F., Séveno, M., Morisset-Lopez, S., Mannoury la Cour, C., Millan, M.J., Bockaert, J., Marin, P., Chaumont-Dubel, S., 2014. Cdk5 induces constitutive activation of 5-HT6 receptors to promote neurite growth. Nat. Chem. Biol. 10, 590–597. https://doi.org/10.1038/nchembio.1547.

Dupuis, A., Wattiez, A.-S., Pinguet, J., Richard, D., Libert, F., Chalus, M., Aissouni, Y., Sion, B., Ardid, D., Marin, P., Eschalier, A., Courteix, C., 2017. Increasing spinal 5-HT2A receptor responsiveness mediates anti-allodynic effect and potentiates fluoxetine efficacy in neuropathic rats. Evidence for GABA release. Pharmacol. Res. 118, 93–103. https://doi.org/10.1016/j.phrs.2016.09.021.

Ennaceur, A., Delacour, J., 1988. A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. Behav. Brain Res. 31, 47–59.

Finnerup, N.B., Attal, N., Haroutounian, S., McNicol, E., Baron, R., Dworkin, R.H., Gilron, I., Haanpaa, M., Hansson, P., Jensen, T.S., Kamerman, P.R., Lund, K., Moore, A., Raja, S.N., Rice, A.S., Rowbotham, M., Sena, E., Siddall, P., Smith, B.H., Wallace, M., 2015. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol. 14, 162–173. https://doi.org/10.1016/S1474-4422(14)70251-0.

Fischer, L., Torres-Chávez, K.E., Clemente-Napimoga, J.T., Jorge, D., Arsati, F., de Arruda Veiga, M.C.F., Tambeli, C.H., 2008. The influence of sex and ovarian hormones on temporomandibular joint nociception in rats. J. Pain 9, 630–638. https://doi.org/10.1016/j.jpain.2008.02.006.

Geranton, S.M., Jimenez-Diaz, L., Torsney, C., Tochiki, K.K., Stuart, S.A., Leith, J.L., Lumb, B.M., Hunt, S.P., 2009. A rapamycin-sensitive signaling pathway is essential for the full expression of persistent pain states. J. Neurosci. 29, 15017–15027. https://doi.org/10.1523/JNEUROSCI.3451-09.2009.

Godinez-Chaparro, B., Lopez-Santillan, F.J., Orduna, P., Granados-Soto, V., 2012. Secondary mechanical allodynia and hyperalgesia depend on descending facilitation mediated by spinal 5-HT(4), 5-HT(6) and 5-HT(7) receptors. Neuroscience 222, 379–391. https://doi.org/10.1016/j.neuroscience.2012.07.008.

Gravius, A., Laszy, J., Pietraszek, M., Sághy, K., Nagel, J., Chambon, C., Wegener, N., Valastro, B., Danysz, W., Gyertyán, I., 2011. Effects of 5-HT6 antagonists, Ro-4368554 and SB-258585, in tests used for the detection of cognitive enhancement and antipsychotic-like activity. Behav. Pharmacol. 22, 122–135. https://doi.org/10.1097/FBP.0b013e328343d804.

Grychowska, K., Satała, G., Kos, T., Partyka, A., Colacino, E., Chaumont-Dubel, S., Bantreil, X., Wesołowska, A., Pawłowski, M., Martinez, J., Marin, P., Subra, G., Bojarski, A.J., Lamaty, F., Popik, P., Zajdel, P., 2016. Novel 1 *H* -pyrrolo[3,2- *c*] quinoline based 5-HT $_6$ receptor antagonists with potential application for the Treatment of Cognitive Disorders Associated with Alzheimer's disease. ACS Chem. Neurosci. 7, 972–983. https://doi.org/10.1021/acschemneuro.6b00090.

He, W., Zhang, B., Zhao, W., He, J., Zhang, L., Xiong, Q., Wang, J., Wang, H., 2019. Contributions of mTOR activation-mediated upregulation of synapsin II and neurite outgrowth to Hyperalgesia in STZ-Induced diabetic rats. ACS Chem. Neurosci. https://doi.org/10.1021/acschemneuro.8b00680.

Hirst, W.D., Abrahamsen, B., Blaney, F.E., Calver, A.R., Aloj, L., Price, G.W., Medhurst, A.D., 2003. Differences in the central nervous system distribution and pharmacology of the mouse 5-hydroxytryptamine-6 receptor compared with rat and human receptors investigated by radioligand binding, site-directed mutagenesis, and molecular modeling. Mol. Pharmacol. 64, 1295–1308. https://doi.org/10.1124/mol.64.6.1295.

Hirst, W.D., Stean, T.O., Rogers, D.C., Sunter, D., Pugh, P., Moss, S.F., Bromidge, S.M., Riley, G., Smith, D.R., Bartlett, S., Heidbreder, C.A., Atkins, A.R., Lacroix, L.P., Dawson, L.A., Foley, A.G., Regan, C.M., Upton, N., 2006. SB-399885 is a potent, selective 5-HT6 receptor antagonist with cognitive enhancing properties in aged rat water maze and novel object recognition models. Eur. J. Pharmacol. 553, 109–119. https://doi.org/10.1016/j.ejphar.2006.09.049.

Hong, J.R., Choo, H., Nam, G., 2017. Neuropathic pain-alleviating effects of pyrazole-conjugated arylsulfonamides as 5-HT6 receptor antagonists. Bioorg. Med. Chem. Lett. 27, 4146–4149. https://doi.org/10.1016/j.bmcl.2017.07.031.

Hunter, J.C., Gogas, K.R., Hedley, L.R., Jacobson, L.O., Kassotakis, L., Thompson, J., Fontana, D.J., 1997. The effect of novel anti-epileptic drugs in rat experimental models of acute and chronic pain. Eur. J. Pharmacol. 324, 153–160. https://doi.org/10.1016/S0014-2999(97)00070-8.

Jiang, L.I., Collins, J., Davis, R., Lin, K.-M., DeCamp, D., Roach, T., Hsueh, R., Rebres, R.A., Ross, E.M., Taussig, R., Fraser, I., Sternweis, P.C., 2007. Use of a cAMP BRET Sensor to Characterize a Novel Regulation of cAMP by the Sphingosine 1-Phosphate/G_{13} Pathway. J. Biol. Chem. 282, 10576–10584. https://doi.org/10.1074/jbc.M609695200.

Khoury, R., Grysman, N., Gold, J., Patel, K., Grossberg, G.T., 2018. The role of 5 HT6-receptor antagonists in Alzheimer's disease: an update. Expert Opin. Investig. Drugs 27, 523–533. https://doi.org/10.1080/13543784.2018.1483334.

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8, e1000412. https://doi.org/10.1371/journal.pbio.1000412.

Kim, S.H., Chung, J.M., 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50, 355–363.

Kohen, R., Fashingbauer, L.A., Heidmann, D.E., Guthrie, C.R., Hamblin, M.W., 2001. Cloning of the mouse 5-HT6 serotonin receptor and mutagenesis studies of the third cytoplasmic loop. Brain Res. Mol. Brain Res. 90, 110–117. https://doi.org/10.1016/s0169-328x(01)00090-0.

Lesiak, A.J., Brodsky, M., Cohenca, N., Croicu, A.G., Neumaier, J.F., 2018. Restoration of Physiological Expression of 5-HT6 Receptor into the Primary Cilia of Null Mutant Neurons Lengthens Both Primary Cilia and Dendrites. Mol. Pharmacol. 94, 731–742. https://doi.org/10.1124/mol.117.111583.

Ling, B., Coudoré-Civiale, M.-A., Balayssac, D., Eschalier, A., Coudoré, F., Authier, N., 2007. Behavioral and immunohistological assessment of painful neuropathy induced by a single oxaliplatin injection in the rat. Toxicology 234, 176–184. https://doi.org/10.1016/j.tox.2007.02.013.

Liu, X.J., Gingrich, J.R., Vargas-Caballero, M., Dong, Y.N., Sengar, A., Beggs, S., Wang, S.-H., Ding, H.K., Frankland, P.W., Salter, M.W., 2008. Treatment of inflammatory and neuropathic pain by uncoupling Src from the NMDA receptor complex. Nat. Med. 14, 1325–1332. https://doi.org/10.1038/nm.1883.

Loiseau, F., Dekeyne, A., Millan, M.J., 2008. Pro-cognitive effects of 5-HT6 receptor antagonists in the social recognition procedure in rats: implication of the frontal cortex. Psychopharmacology (Berl.) 196, 93–104. https://doi.org/10.1007/s00213-007-0934-5.

Meffre, J., Chaumont-Dubel, S., Mannoury la Cour, C., Loiseau, F., Watson, D.J., Dekeyne, A., Seveno, M., Rivet, J.M., Gaven, F., Deleris, P., Herve, D., Fone, K.C., Bockaert, J., Millan, M.J., Marin, P., 2012. 5-HT(6) receptor recruitment of mTOR as a mechanism for perturbed cognition in schizophrenia. EMBO Mol. Med. 4, 1043–1056. https://doi.org/10.1002/emmm.201201410.

Mestre, C., Pelissier, T., Fialip, J., Wilcox, G., Eschalier, A., 1994. A method to perform direct transcutaneous intrathecal injection in rats. J. Pharmacol. Toxicol. Methods 32, 197–200.

Millan, M.J., 2002. Descending control of pain. Prog. Neurobiol. 66, 355–474.

Nicholson, R., Small, J., Dixon, A.K., Spanswick, D., Lee, K., 2003. Serotonin receptor mRNA expression in rat dorsal root ganglion neurons. Neurosci. Lett. 337, 119–122.

Obara, I., Tochiki, K.K., Géranton, S.M., Carr, F.B., Lumb, B.M., Liu, Q., Hunt, S.P., 2011.

Systemic inhibition of the mammalian target of rapamycin (mTOR) pathway reduces neuropathic pain in mice. Pain 152, 2582–2595. https://doi.org/10.1016/j.pain.2011.07.025.

Pastrana-Quintos, T., Salgado-Moreno, G., Pérez-Ramos, J., Coen, A., Godínez-Chaparro, B., 2020. Anti-allodynic effect induced by curcumin in neuropathic rat is mediated through the NO-cyclic-GMP-ATP sensitive K + channels pathway. BMC Complement Med Ther 20, 83. https://doi.org/10.1186/s12906-020-2867-z.

Pezet, S., 2014. Neurotrophines et douleur. Biol. Aujourdhui 208, 21–29. https://doi.org/10.1051/jbio/2014002.

Pichon, X., Wattiez, A.S., Becamel, C., Ehrlich, I., Bockaert, J., Eschalier, A., Marin, P., Courteix, C., 2010. Disrupting 5-HT(2A) receptor/PDZ protein interactions reduces hyperalgesia and enhances SSRI efficacy in neuropathic pain. Mol. Ther. 18, 1462–1470 https://doi.org/w.

Pierce, P.A., Xie, G.X., Meuser, T., Peroutka, S.J., 1997. 5-Hydroxytryptamine receptor subtype messenger RNAs in human dorsal root ganglia: a polymerase chain reaction study. Neuroscience 81, 813–819.

Pineda-Farias, J.B., Barragán-Iglesias, P., Valdivieso-Sánchez, A., Rodríguez-Silverio, J., Flores-Murrieta, F.J., Granados-Soto, V., Rocha-González, H.I., 2017. Spinal 5-HT4 and 5-HT6 receptors contribute to the maintenance of neuropathic pain in rats. Pharmacol. Rep. 69, 916–923. https://doi.org/10.1016/j.pharep.2017.04.001.

Radat, F., Margot-Duclot, A., Attal, N., 2013. Psychiatric co-morbidities in patients with chronic peripheral neuropathic pain: a multicentre cohort study. Eur. J. Pain 17, 1547–1557. https://doi.org/10.1002/j.1532-2149.2013.00334.x.

Randall, L.O., Selitto, J.J., 1957. A method for measurement of analgesic activity on inflamed tissue. Arch. Int. Pharmacodyn. Ther. 111, 409–419.

Sari, C.C., Gunduz, O., Ulugol, A., 2019. Spinal serotonin and 5HT6 receptor levels during development of neuropathy and influence of blockade of these receptors on thermal hyperalgesia in diabetic mice. Drug Res (Stuttg). https://doi.org/10.1055/a-0817-5464.

Seretny, M., Currie, G.L., Sena, E.S., Ramnarine, S., Grant, R., MacLeod, M.R., Colvin, L.A., Fallon, M., 2014. Incidence, prevalence, and predictors of chemotherapy-induced peripheral neuropathy: a systematic review and meta-analysis. Pain 155, 2461–2470. https://doi.org/10.1016/j.pain.2014.09.020.

Teng, L.-L., Lu, G.-L., Chiou, L.-C., Lin, W.-S., Cheng, Y.-Y., Hsueh, T.-E., Huang, Y.-C., Hwang, N.-H., Yeh, J.-W., Liao, R.-M., Fan, S.-Z., Yen, J.-H., Fu, T.-F., Tsai, T.-F., Wu, M.-S., Wang, P.-Y., 2019. Serotonin receptor HTR6-mediated mTORC1 signaling regulates dietary restriction–induced memory enhancement. PLoS Biol. 17, e2007097. https://doi.org/10.1371/journal.pbio.2007097.

Treede, R.-D., Jensen, T.S., Campbell, J.N., Cruccu, G., Dostrovsky, J.O., Griffin, J.W., Hansson, P., Hughes, R., Nurmikko, T., Serra, J., 2008. Neuropathic pain: redefinition and a grading system for clinical and research purposes. Neurology 70, 1630–1635. https://doi.org/10.1212/01.wnl.0000282763.29778.59.

Uttam, S., Wong, C., Price, T.J., Khoutorsky, A., 2018. eIF4E-dependent translational control: a central mechanism for regulation of pain plasticity. Front. Genet. 9, 470. https://doi.org/10.3389/fgene.2018.00470.

van Hecke, O., Austin, S.K., Khan, R.A., Smith, B.H., Torrance, N., 2014. Neuropathic pain in the general population: a systematic review of epidemiological studies. Pain 155, 654–662. https://doi.org/10.1016/j.pain.2013.11.013.

Vanda, D., Soural, M., Canale, V., Chaumont-Dubel, S., Satała, G., Kos, T., Funk, P., Fülöpová, V., Lemrová, B., Koczurkiewicz, P., Pękala, E., Bojarski, A.J., Popik, P., Marin, P., Zajdel, P., 2018. Novel non-sulfonamide 5-HT6 receptor partial inverse agonist in a group of imidazo[4,5-b]pyridines with cognition enhancing properties. Eur. J. Med. Chem. 144, 716–729. https://doi.org/10.1016/j.ejmech.2017.12.053.

Wang, L., Lv, Y., Deng, W., Peng, X., Xiao, Z., Xi, Z., Chen, G., Wang, X., 2015. 5-HT6 receptor recruitment of mTOR modulates seizure activity in epilepsy. Mol. Neurobiol. 51, 1292–1299. https://doi.org/10.1007/s12035-014-8806-6.

Wang, X., Li, X., Huang, B., Ma, S., 2016. Blocking mammalian target of rapamycin (mTOR) improves neuropathic pain evoked by spinal cord injury. Transl. Neurosci. 7, 50–55. https://doi.org/10.1515/tnsci-2016-0008.

Ward, R.P., Dorsa, D.M., 1996. Colocalization of serotonin receptor subtypes 5-HT2A, 5-HT2C, and 5-HT6 with neuropeptides in rat striatum. J. Comp. Neurol. 370, 405–414. https://doi.org/10.1002/(SICI)1096-9861(19960701)370:3&405::AID-CNE10&3.0.CO;2-R.

Zajdel, P., Marciniec, K., Satała, G., Canale, V., Kos, T., Partyka, A., Jastrzębska-Więsek, M., Wesołowska, A., Basińska-Ziobroń, A., Wójcikowski, J., Daniel, W.A., Bojarski, A.J., Popik, P., 2016. N1-azinylsulfonyl-1H-indoles: 5-HT6 receptor antagonists with procognitive and antidepressant-like properties. ACS Med. Chem. Lett. 7, 618–622. https://doi.org/10.1021/acsmedchemlett.6b00056.

Zhang, W., Sun, X.F., Bo, J.H., Zhang, J., Liu, X.J., Wu, L.P., Ma, Z.L., Gu, X.P., 2013. Activation of mTOR in the spinal cord is required for pain hypersensitivity induced by chronic constriction injury in mice. Pharmacol. Biochem. Behav. 111, 64–70. https://doi.org/10.1016/j.pbb.2013.07.017.