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Multiple sites and actions of gabapentin-induced relief of ongoing experimental neuropathic pain

Kirsty Bannister[2], **Chaoling Qu**[1], **Edita Navratilova**[1], **Janice Oyarzo**[1], **Jennifer Yanhua Xie**[1], **Tamara King**[3], **Anthony H. Dickenson**[2], and **Frank Porreca**[1]

[1]Department of Pharmacology, Arizona Health Sciences Center, University of Arizona, Tucson, AZ 85724, USA

[2]Neuroscience, Physiology and Pharmacology, University College London, London, WC1E 6BT, United Kingdom

[3]Department of Biomedical Sciences, Center for Excellence in the Neurosciences, University of New England, Biddeford, ME 04005, USA

Abstract

Gabapentin is a first-line therapy for neuropathic pain but its mechanisms and sites of action remain uncertain. We investigated gabapentin-induced modulation of neuropathic pain following spinal nerve ligation (SNL) in rats. Intravenous or intrathecal gabapentin reversed evoked mechanical hypersensitivity, produced conditioned place preference (CPP) and dopamine release in the nucleus accumbens (NAc) selectively in SNL rats. Spinal gabapentin also significantly inhibited dorsal horn wide dynamic range (WDR) neuronal responses to a range of evoked stimuli in SNL rats. In contrast, gabapentin microinjected bilaterally into the rostral anterior cingulate cortex (rACC), produced CPP and elicited NAc dopamine release selectively in SNL rats but did not reverse tactile allodynia and had marginal effects on WDR neuronal activity. Moreover, blockade of endogenous opioid signaling in the rACC prevented intravenous gabapentin-induced CPP and NAc dopamine release but failed to block its inhibition of tactile allodynia. Gabapentin therefore can potentially act to produce its pain relieving effects by (a) inhibition of injury-induced spinal neuronal excitability, evoked hypersensitivity and ongoing pain and (b) selective supraspinal modulation of affective qualities of pain, without alteration of reflexive behaviors. Consistent with previous findings of pain relief from non-opioid analgesics, gabapentin requires engagement of rACC endogenous opioid circuits and downstream activation of mesolimbic reward circuits reflected in learned pain motivated behaviors. These findings support the partial separation of sensory and affective dimensions of pain in this experimental model and suggest that modulation of affective-motivational qualities of pain may be the preferential mechanism of gabapentin's analgesic effects in patients.

Corresponding Author: Frank Porreca, Ph.D., Department of Pharmacology, University of Arizona, Tucson, AZ 85724, (520) 626-7421 (voice), (520) 626-4182 (fax), frankp@u.arizona.edu.

Conflict of interest statement
The authors have no conflict of interest to declare.

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Anterior cingulate cortex; endogenous opioids; spinal cord; neuropathic pain; pain affect; gabapentin

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1 Introduction

Effective treatments for chronic neuropathic pain remain an important unmet medical need. Currently available medications achieve clinically significant pain relief in only about 40-60% patients [15]. Neuropathic pain can result from injury or disease of the nervous system [7] and is variably characterized by allodynia and hyperalgesia (i.e., enhanced response to normally innocuous or noxious stimuli, respectively). Many patients also suffer from ongoing pain that is independent of external evoked stimuli [2; 54]. Ongoing pain diminishes patients' quality of life, and causes them to seek medical attention for pain relief [2; 54].

Gabapentinoids, including pregabalin and gabapentin (GBP), are first-line treatments for neuropathic pain but their mechanisms and sites of action remain uncertain [41; 45; 55; 66]. Gabapentin demonstrates higher efficacy for neuropathic, than postoperative, pain with numbers needed to treat to benefit (NNTs) of 5.8 and 11, respectively [41; 56]. In neuropathic pain patients, gabapentin alleviated ongoing pain, but had no effect on pain thresholds to mechanical and heat stimuli [1]. In preclinical neuropathic pain models, gabapentinoids effectively reversed tactile and thermal allodynia, but produced negligible effects on acute nociception [29]. Studies in $\alpha_2\delta$-1 knock-out and mutant mice demonstrated that the anti-allodynic effects of gabapentinoids required this subunit of voltage gated calcium channels [19; 48]. Preclinical findings suggest that gabapentinoids may produce anti-allodynic effects in the spinal cord by inhibiting neurotransmitter release [18; 40].

A clinical trial with chronic pain patients, however, found no analgesic effects of intrathecal gabapentin infusion [53] suggesting that supraspinal sites may be necessary. Indeed, neuroimaging in heathy subjects with experimental capsaicin-induced hyperalgesia identified brain regions where gabapentin reduced BOLD activity in response to mechanical stimulation of the sensitized skin including the anterior cingulate, insular, and secondary somatosensory cortices, thalamus and the brainstem [31; 64]. The involvement of supraspinal sites was also demonstrated in fibromyalgia patients, where reduction in the pain assessment following oral gabapentin treatment for 14 days correlated with reduced glutamate levels in the insula and decreased insular connectivity with the default mode network [25].

Pain is an unpleasant experience with both sensory and affective dimensions. Previous rodent studies have shown that the aversiveness of pain, and its relief, can be assessed independently of reflexive measures with conditioned place preference/aversion (i.e., CPP/CPA) testing [32; 33; 35]. Importantly, these are learning paradigms that depend on pain processing in the brain. Using this approach, intrathecal clonidine, an α_2 adrenergic receptor agonist effective in humans, produced CPP selectively in rodent experimental models of neuropathic pain (e.g., spinal nerve ligation, SNL) [35]. Clonidine, and other non-opioid

pain relieving treatments, also increased extracellular dopamine (DA) levels in the nucleus accumbens (NAc) [67]. The reward of pain relief and activation of the mesolimbic dopamine pathway has been shown in multiple models of ongoing pain [42; 44]. Here, we used CPP and NAc DA release as measures of relief of pain-related aversiveness along with evaluation of evoked hypersensitivity and spinal cord electrophysiology to investigate the effects of gabapentin at spinal and supraspinal sites on affective and sensory aspects of pain.

2 Materials and Methods

2.1 Animals

Male, Sprague Dawley rats (Harlan) weighing 250-350 g at time of testing, were maintained in a climate-controlled room on a 12 h light/dark cycle, and food and water were available ad libitum. All experiments were performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals and received approval from the Institutional Animal Care and Use Committee of the University of Arizona, AZ or by Biological Services, UCL, UK. All efforts were made to minimize animal suffering and reduce the number of animals used. All behavioral experiments were carried out by investigators blinded to the treatment groups.

2.2 Surgical procedures

2.2.1 Intracranial rACC cannulation—Animals were anesthetized with a ketamine (80 mg/kg; i.p.; Western Medical Supply) and xylazine (12 mg/kg; i.p.; Sigma) mixture and placed in a stereotaxic apparatus. Bilateral cannulation of the rACC was performed as previously described [32; 43]. A pair of stainless steel guides (33-gauge) were implanted 1 mm above the rACC injection site (anteroposterior: bregma +2.6 mm; lateral ± 0.6 mm; dorsoventral: skull -1.6 mm; [51]). Guide cannulas were cemented in place and secured to the skull by small stainless steel machine screws. Stainless steel dummy cannulas extending to the tip of the guide cannulas were inserted to keep the guide free of debris until drug delivery. Rats then received subcutaneous injection of gentamycin (1 mg/ml) and were allowed to recover for 7-10 days. Subsequently, the rats received SNL or sham surgeries. No signs of weight loss or distress were observed following cannulation surgeries.

2.2.2 Intrathecal (i.th.) cannulation—Animals were anesthetized with an i.p. injection of a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg) and placed in a stereotaxic apparatus. The atlanto-occipital membrane was exposed, cleared and an incision was made in the dura mater. A length of PE-10 tubing was advanced 7.5 cm caudally to the lumbar spinal cord. The tubing was exteriorized, filled with saline and heat sealed. The wound was closed and rats then received subcutaneous gentamycin (1 mg/ml) injection and were allowed to recover for 7-10 days before any subsequent SNL or sham surgeries and behavioral testing.

2.2.3 Spinal nerve ligation—The surgical procedure for L_5/L_6 SNL was performed according to Kim and Chung [34]. Anesthesia was induced with 5% and maintained with 2% isofluorane in air. A 2 cm midline incision was made in the skin at the level of the hip

bone and the transverse process at L_5 was removed. The L_5 and L_6 spinal nerves were tightly ligated with 4-O silk suture. The incision was closed and the animals received subcutaneous gentamycin (1 mg/ml) injection. Sham-operated control rats were prepared in an identical manner except that the L_5/L_6 spinal nerves were not ligated. Rats were allowed to recover for 10-12 days. The behavior of the rats was monitored carefully for any visual indication of motor disorders or change in weight or general health. Rats that exhibited motor deficiency or that failed to develop tactile allodynia were excluded from further testing.

2.2.4 NAc microdialysis cannulation and dual NAc/rACC cannulation—All stereotaxic surgeries were performed in rats anesthetized with a ketamine/xylazine mixture (80/120 mg/kg, i.p.) and cannulas were implanted according to the Paxinos brain atlas [51] to perform the microdialysis experiments [67]. For NAc microdialysis with intravenous or intrathecal treatments, a single guide cannula (AG-8; Eicom Corp.) was implanted vertically into the left NAc shell: (AP: bregma +1.7 mm; ML: midline +1.0 mm; DV: skull -6.0 mm). For microdialysis studies requiring bilateral rACC injections, 26-guage guide cannulas (Plastics One) were implanted into the rACC at a 25° forward facing angle (AP: bregma +4.1 mm; ML: midline ±0.8; DV: -3.0 mm) together with the microdialysis NAc guide cannula (AP: bregma +1.7 mm; ML: midline +1.0 mm; DV: skull -6.0 mm). After surgery, rats received subcutaneous gentamycin (1 mg/ml) injection. All animals were housed individually and allowed a minimum of 7 days to recover.

2.3 Drug administration

All injections were delivered in a separate room to avoid any possible effects of injection-induced stress vocalizations on the CPP behaviors of the rats. Gabapentin was dissolved in distilled water. Systemic GBP was administrated by intravenous injection (50 mg/kg). Human dosing of Neurontin (300-600 mg, p.o.) corresponds to approximately 5-10 mg/kg. We used a dose 5-10 fold higher to account for faster rate of elimination in rats [61; 65]. This dose is based on previous studies that used intraperitoneal or subcutaneous dosing of 100 mg/kg in rats [17; 23; 24; 38] At this dose, gabapentin did not produce sedation [23]. Moreover, i.p. administration of gabapentin at doses up to 300 mg/kg in rat did not affect heart rate and blood pressure [68]. For spinal drug administration, 5 μl of saline or gabapentin (200 μg/5 μl) [20; 68] were injected slowly via the intrathecal catheter, followed by a 1 μl air bubble and 9 μl saline flush. Clinical studies with continuous intrathecal administration of gabapentin in patients with intractable non-cancer pain used doses ranging from 1-30 mg/day [53]. Based simply on body weight this would translate to 5-150 μg in rats. Microinjections into the rACC were performed through injectors protruding 1 mm beyond the guide cannula tip by slowly expelling 0.5 μl volume of gabapentin (100 μg/0.5 μl) or vehicle across a 1 min period. This dose was implemented from the mouse study [57]. Gabapentin was purchased from Spectrum Chemical MFG (Gardena, CA). β-funaltrexamine (β-FNA) was purchased from Tocris (Ellisville, MO). Cocaine was obtained from NIDA drug supply program.

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2.4 Behavioral testing

2.4.1 Tactile hypersensitivity—The withdrawal threshold of the hindpaw was measured in response to probing of the plantar surface with a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) in logarithmically spaced increments ranging from 0.41 to 15 g (4–150 N). Each filament was applied perpendicularly to the plantar surface of the left hindpaw of rats kept in suspended wire-mesh cages. Withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength ("up and down" method), analyzed using a Dixon nonparametric test, and expressed as the mean withdrawal threshold [14].

2.4.2 Thermal hypersensitivity—The withdrawal latency of the hindpaw to an infrared radiant heat source was performed as previously described [8]. Baseline latencies were established at 17-25 s to allow a sufficient window for the detection of possible hyperalgesia. A maximal cutoff of 33 s was used to prevent tissue damage.

2.4.3 Conditioned place preference—The single trial conditioned place preference protocol was performed as previously described. [52] On pre-conditioning day (Day 1), 13 days after either SNL or sham surgery, rats were placed into the CPP boxes with access to all chambers and time spent in each chamber over 15 min was determined by an automated process. To assure no chamber preference bias prior to conditioning, animals spending more than 80% (720 s) or less than 20% (180 s) of the total time in a chamber were eliminated from further testing. Chamber pairings were counterbalanced between the control and drug chambers. The following day (Day 2), all rats received vehicle (saline) injections either i.v. (1 ml/kg), i.th. (5 μl), or by microinjection into the rACC (0.5 μl/side; bilateral) and were immediately placed into one randomly-assigned conditioning chamber for 30 min without access to the other chamber. Four hours later in the afternoon, rats received the corresponding gabapentin treatment either i.v. (50 mg/kg, 1 ml/kg), i.th. (200 μg/5 μl), or into the rACC (100 μg/0.5 μl/side; bilateral) paired with the opposite chamber for 30 minutes. On test day (Day 3), 20 hours following the afternoon pairing, rats were placed in the CPP box with access to all chambers again and their behavior was recorded for 15 minutes for analysis of chamber preference. The CPP test is a learning paradigm where kinetics of drug administration can influence the outcome of single trial conditioning. We used i.v. gabapentin to promote rapid effects that allow direct pairing of GBP treatment with the CPP chamber, even though this drug is given orally in humans [41]. In experiments involving blockade of opioid signaling in the rACC, after the baseline testing on day 1, the rats received bilateral injections of either vehicle (saline, 1 μl/side) or a selective irreversible μ-opioid receptor antagonist β-funaltrexamine (β-FNA 3 μg/1 μl/side) into the rACC and then underwent conditioning and testing on days 2 and 3 as described. A total of 146 rats were used for all CPP experiments; 59 animals were excluded from the studies due to either 1) lack of development of chronic pain, 2) paralysis following i.th catheter, 3) CPP chamber bias during baseline or 4) post-hoc due to incorrect cannula placement.

2.5 *In vivo* microdialysis and HPLC quantification of dopamine

Microdialysis was done in awake, freely moving animals [67]. The microdialysis probe (AI-8-2, EICOM, San Diego, CA) was inserted into the NAc with 2-mm semipermeable

membrane (MW cutoff: 20 kDa) projecting beyond the guide cannula and perfused at 1.25 μl/min with artificial cerebrospinal fluid (aCSF: 147.0 mM NaCl, 2.8 mM KCl, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$). After a 90-minute washout period, a 90 min baseline fraction was collected into prechilled (4°C) amber Eppendorf tubes containing 1.0 μl 40x antioxidant solution (6.0 mM L-cysteine, 2.0 mM oxalic acid, and 1.3% w/v glacial acetic acid) [28]. Rats were then treated with the appropriate drug and a 90 min experimental fraction was collected. In experiments involving β-FNA pretreatment, β-FNA was administrated into the rACC one day prior to the microdialysis experiment. After testing, all rats were injected with cocaine (20 mg/kg, i.p.) and dialysates were collected for an additional 60 minutes.

Fractions were analyzed using Agilent 1100 HPLC system (Agilent, USA) with a 5020 guard cell, MD-150 column, and Coulochem III 5014B electrochemical detector (Thermofisher; USA) at ambient temperature. The guard cell was set at 350 mV, Electrode1 at -150 mV and Electrode2 at 250 mV. A standard curve was produced from 6 serial dilutions of DA (1.25-40 pg) in 20 μl aCSF plus antioxidant cocktail. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the following formulas: LOD = 3.3 (SDr/S); LOQ = 10 (SDr/S), where the standard deviation of the response SDr (SD of y-intercepts of regression lines) and the slope of the standard curve S were determined from the measurements of 10 independent standard curves. The LOD and LOQ for DA were determined to be 0.286 and 0.868 pg on column, respectively. The linearity of DA peaks was also validated. The integration of the DA peaks from HPLC chromatograms was performed by an experimenter blinded to the treatment groups. DA concentrations in the microdialysate samples were expressed as picograms per microliter. The percent change from the corresponding baseline level was calculated to normalize the variations of individual rats and to allow for multiple comparisons. Rats that had basal DA levels below the limit of quantification (LOQ) in the dialysates, or that failed to demonstrate an increase of >100% over baseline levels after cocaine administration, suggesting insufficient permeation through the microdialysis membrane, were excluded from data analysis post hoc. Ninety-six rats were used in total for the microdialysis experiments; 28 animals were excluded from the studies due to 1) the lack of allodynia in SNL rats, 2) paralysis following i.th catheterization or 3) incorrect permeation or location of the microdialysis probe.

2.6 Electrophysiology

In vivo electrophysiology experiments were conducted on post-operative days 14-18 (sham and SNL-operated animals) or weight/age matched naive rats as previously described [59]. Animals were anesthetized and maintained for the duration of the experiment with isoflurane (1.5%) delivered in a gaseous mix of N_2O (66%) and O_2 (33%). Animals were secured to a stereotaxic frame. A laminectomy was performed to expose the L4-5 segments of the spinal cord. Extracellular recordings were made from deep dorsal horn neurons (lamina V-VI) using 2 MΩ 127 μm diameter parylene coated tungsten electrodes (A-M systems, Sequim, WA). All the neurons recorded were wide dynamic range (WDR) and responded to natural stimuli including brush, low and high intensity mechanical and thermal stimuli in a graded manner with coding of increasing intensity.

The receptive fields of the neurons were on the ipsilateral hindpaw within the sciatic nerve territory, typically two toes extending into the plantar area. A train of 16 transcutaneous electrical stimuli (2 ms width and 0.5 Hz), delivered via stimulating needles inserted into the center of the peripheral receptive field, was applied at 3 times the threshold current for C-fiber activation of the dorsal horn WDR cell (typically around 3 mA). A post-stimulus histogram was constructed and responses evoked by Aβ- (0-20 ms), Aδ- (20-90 ms) and C-fibers (90-350 ms) were separated and quantified on the basis of latency. Neuronal responses occurring after the C-fiber latency band were quantified as post-discharge (350-800 ms). Activity dependent hyper-excitability was measured as 'wind-up', calculated as the difference between the total number of action potentials at C-fiber latency produced by the train of 16 electrical stimuli, and 'input', which represented the post-synaptic C-fiber-evoked dorsal horn neuronal response following the first of the 16 electrical stimuli in the electrical train.

The peripheral receptive field was stimulated using mechanical stimuli (brush and von Frey filaments 2, 8, 26 and 60 g) and thermal stimuli (42, 45 and 48°C applied with a constant water jet). All natural stimuli were applied for 10 s each. Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design; PSTH and rate functions).

Three baseline responses to peripheral stimuli as detailed above were characterized for each neuron before pharmacological assessment (a drug study was carried out on one neuron per animal only) following 3 consecutive stable control trials (10% variation for C-fiber evoked, <20% variation for all other parameters). Neuron values were averaged to give the pre-drug control values. Following collection of baseline control data gabapentin (BIONET research, Cornwall, UK, 10μg/0.5μl, dissolved in saline) was micro-injected into the rACC. For spinal application, 50 μl of gabapentin (200 μg/50 μl) was applied slowly to the exposed spinal cord well. Each individual drug dose effect was followed for up to 60 minutes with tests carried out at 10, 30 and 60 minutes. For the post-drug effects, maximal changes from pre-drug baseline values are plotted. Ten rats were used for electrophysiological experiments (5 rats per experiment); no animals were excluded from the studies.

2.7 Verification of rACC cannula placements

Following the experiments, rats were euthanized and 0.5 μl India ink was injected into the rACC cannulas in the same manner as drug delivery. Brains were removed and post-fixed in 10% formalin for a minimum of 4 h. Brains were frozen and sliced at 30 μm, and the location of the ink recorded. Animals with incorrectly placed cannulas were excluded from the data analysis.

2.8 Statistical analysis

For analysis of evoked pain behaviors, two-way ANOVA followed by *post-hoc* Tukey's Multiple Comparison Test was performed. For CPP experiments, the difference score, obtained by taking the difference between the pre-conditioning (baseline) and post-conditioning (test) time spent in the paired chamber were examined by Student's paired t-test (Excel, Microsoft office). For microdialysis experiments, NAc DA levels are expressed

as percent of their corresponding baseline levels for individual rats. An unpaired t-test (two-tailed) was used to compare the changes of dopamine level in NAc shell after different treatments. All evaluations were obtained using GraphPad Prism 5 software (San Diego, CA). For electrophysiological experiments, statistical analyses were performed using SPSSV22 (IBM, Armonk, NY). Statistical differences in fiber threshold, electrical parameters and neuronal responses to dynamic brush stimulation were determined using a paired Students t-test. Differences in mechanical and thermal coding were determined using a 2-way repeated-measures ANOVA followed by Bonferroni correction for multiple paired comparisons. Sphericity was tested with Mauchly's test. All data represents mean ± SEM. Significance was set at $p<0.05$. Asterisks denote statistically significant differences ($*P<0.05$).

3 Results

3.1 Intravenous gabapentin reduces both evoked and ongoing pain in SNL rats promoting pain relief-motivated behavior

The analgesic effects of intravenously administered gabapentin on ongoing neuropathic pain were investigated in rats following spinal nerve ligation (SNL). This model of nerve injury-induced pain is characterized by mechanical and thermal hypersensitivity as well as by ongoing pain that can last for at least 60 days [63]. Mechanical allodynia observed in SNL rats 14-21 days after the surgery was reversed by i.v. gabapentin (50 mg/kg) in a time-dependent manner (sham: 7; SNL: n=9; Fig. 1A); the peak effect was seen 20 min after administration. In the CPP test, this dose of gabapentin also produced significant preference for the chamber paired with the drug, revealing gabapentin's efficacy in relieving ongoing neuropathic pain (difference score = 141±50 s; n=17; p=0.01; Fig. 1B). Importantly, sham operated rats showed no preference for the drug-paired chamber (difference score = 38±45 s; n=10; Fig. 1B), verifying that gabapentin is not rewarding in a normal state, and that its rewarding quality in SNL rats is likely due to relief of ongoing aversiveness associated with pain. This interpretation was supported by the outcomes of NAc dopamine release measurements using *in vivo* microdialysis in freely moving rats. Following i.v. gabapentin administration, only SNL rats demonstrated increased dopamine levels in the shell region of the NAc (70±13% increase in SNL (n=12) vs. 6±10% in sham (n=11) rats; $F_{(21)}=1.735$; p=0.0045; Fig. 1C).

3.2 The rewarding/motivational effects of pain relief following i.v. gabapentin are dependent on endogenous opioid signaling in the rACC

Our previous studies suggest that endogenous opioid signaling in the rACC is required for reward from pain relief following opioid and non-opioid treatment [43]. To determine whether gabapentin also relies on this mechanism for its pain relieving effects, we injected an irreversible opioid receptor antagonist β-funaltrexamine (β-FNA; 3 μg/1 μl) or vehicle (saline, 1 μl) into the rACC, and 24 h later measured GBP-induced CPP and NAc dopamine release. Microinjections of saline or β-FNA into the rACC did not have any effects on the anti-allodynic efficacy of i.v. gabapentin in SNL rats (saline: n=4; β-FNA: n=6; Fig. 2A). However, SNL rats that received saline into the rACC showed robust CPP to i.v. gabapentin paired chamber (difference score = 177±61 s; n=7; p<0.01, Fig. 2B), while no significant

CPP was observed in SNL rats pretreated with rACC β-FNA (difference score = 54±30 s; n=12; Fig. 2B). Thus, endogenous opioid signaling in the rACC is required for intravenous GBP-induced pain relief. Similarly, in microdialysis experiments, SNL rats that received saline into the rACC showed significantly increased DA levels in NAc, but rats receiving rACC β-FNA did not show any DA efflux (52±13% increase in saline (n=8) vs. 1±9% in β-FNA (n=9) pretreated rats; $F_{(15)}$=1.731; p=0.0059; Fig. 2C). These data suggest that endogenous opioid signaling in the rACC is required for rewarding actions of i.v. gabapentin-mediated relief, but is not necessary for the anti-allodynic effects on evoked tactile responses. Gabapentin, might therefore exert different analgesic effects by engaging spinal or supraspinal sites. For this reason, we further investigated whether gabapentin can act directly in the rACC to relieve aversiveness or whether these effects are indirect through binding of gabapentin to its targets in the spinal cord.

3.3 Intrathecal actions of gabapentin are sufficient to reduce evoked and ongoing pain in SNL rats

SNL and sham-operated rats received a spinal injection of gabapentin (200 μg) via an intrathecal catheter and paw withdrawal thresholds to tactile stimuli or paw withdrawal latencies to noxious radiant heat were measured at 20, 40, 60, 90 and 120 min after the injection. Rats with SNL showed significant ($p<0.05$) decrease in paw withdrawal thresholds and paw withdrawal latencies to tactile and thermal stimuli, respectively (Fig. 3A, B). Intrathecal gabapentin produced a time-dependent reversal of SNL-induced tactile allodynia observed between 20 and 120 min after gabapentin administration while i.th. saline had no effect (saline: n=9; GBP; n=10; Fig. 3A). SNL-induced thermal hyperalgesia was also transiently reduced by intrathecal gabapentin but not by saline (saline; n=9; GBP; n=10; Fig. 3B). These treatments had no effects on evoked behaviors in animals with sham surgeries (not shown).

In the CPP test, the same dose of i.th. gabapentin produced significant preference for the chamber paired with the treatment, indicating gabapentin's efficacy in relieving ongoing (spontaneous) neuropathic pain (difference score = 194±51 s; n=10; p=0.0041; Fig. 3C). Sham-operated rats did not show any preference for the chamber associated with gabapentin (difference score = -7±57 s; n=12; Fig. 3C). Microdialysis experiments in SNL rats demonstrated elevated levels of dopamine in the NAc following i.th. administration of gabapentin but not following i.th administration of saline (129±62% increase after GBP; n=8 vs. 18±17% following saline; n=7; p=ns; Fig. 3D). Together, these data support a notion that gabapentin can act directly in the spinal cord to inhibit evoked nociceptive signaling and indirectly through supraspinal sites including opioid circuits in the rACC to relieve ongoing pain and motivate behaviors.

3.4 Intrathecally administered gabapentin reduces evoked dorsal horn neuronal responses in SNL rats

We performed *in vivo* electrophysiology in spinal nerve ligated rats (n = 5) and demonstrated that gabapentin applied directly to the spinal cord significantly inhibited wide dynamic range (WDR) neuronal responses to noxious mechanical ($p<0.05$ for 26 and 60 g von Frey forces; 2-way RM-ANOVA; p = 0.022; $F_{(1,4)}$ = 13.29; Fig. 3E) and noxious

thermal ($p<0.05$ for 48°C only; 2-way RM-ANOVA; $p = 0.172$; $F_{(1,4)} = 2.754$; Fig. 3F) stimuli. Further, compared to pre-drug baselines, Aδ- and C-fiber responses, input and brush responses were also significantly reduced ($p = 0.005$; 0.034; 0.036 and 0.000 respectively; paired sample t-Test; Fig. 3G). The maximal change from baseline was observed either at 10 or 30 minutes depending on the animal.

3.5 Administration of gabapentin into the rACC is sufficient to elicit CPP and NAc dopamine release in SNL rats without modulating evoked pain responses

To determine if gabapentin can directly act in the rACC to alleviate aversiveness associated with ongoing pain, we injected vehicle (saline; 0.5 μl/site) or gabapentin (100 μg/0.5 μl/site) into the rACC of SNL or sham rats. In injured rats, neither saline nor gabapentin in the rACC had any effect on nerve ligation-induced tactile allodynia (n=5; Fig. 4A). However, in SNL rats, microinjection of gabapentin into the rACC produced CPP, indicated by significant difference score (189±56 s; n=8; p=0.0116; Fig. 4B). Gabapentin did not produce CPP in sham-operated rats (15±48 s; n=8; p=0.7661; Fig. 4B). Consistent with the CPP data, rACC microinjection of gabapentin significantly increased dopamine levels in the NAc in SNL rats (103±43% increase following GBP (n=5) vs. 8±12% following saline (n=8); $F_{(11)}=7.464$; p=0.026; Fig. 4C). Therefore, gabapentin may have additional supraspinal sites of action including the rACC where it may directly modulate aversive aspects of pain independently of its spinal effects.

3.6 Administration of gabapentin in the rACC has no effect on evoked dorsal horn neuronal responses to natural stimuli and minimal effects on electrically evoked dorsal horn neuronal responses in SNL rats

To investigate if gabapentin action in the rACC could result in the engagement of descending pain modulatory pathways to inhibit neuronal activity at the spinal cord level, we measured electrophysiological responses of WDR neurons following administration of gabapentin into the rACC of SNL rats (n=5). Compared to baseline, gabapentin had no significant effect on evoked WDR neuronal responses to mechanical or thermal stimuli ($p>0.05$ for all natural stimuli; 2-way RM-ANOVA; $p = 0.052$, $F_{(1,4)} = 6.634$ and $p = 0.058$, $F_{(1,4)} = 6.27$, respectively) (Fig. 4D,E). We did however observe that rACC gabapentin had a significantly inhibitory effect on a subset of electrically evoked WDR neuronal responses at 10 or 30 min time point (C-fiber activity and post discharge, $p = 0.043$ and 0.032, respectively; paired sample t-test) (Fig. 4F). Saline micro-injection into the rACC had no effect on WDR neuronal evoked responses to natural or electrical stimuli ($p>0.05$ for all stimuli; 2-way RM-ANOVA; $p = 0.565$, $F_{(1,4)} = 0.393$) (data not shown).

4 Discussion

Gabapentin and pregabalin were designed as analogs of GABA, but they do not bind to either ionotropic or metabotropic GABA receptors. Instead, these drugs exhibit high affinity for the $\alpha_2\delta$-subunits of voltage gated calcium channels [21]. The use of $\alpha_2\delta$-1 knock-out and mutant mice established the requirement of this subunit for the analgesic effects of gabapentinoid drugs presumably by modulation of transmitter release in the spinal cord [19; 48]. It is therefore surprising that gabapentinoids have minimal effects on nociceptive

transmission, and promote analgesia only in states with central sensitization, as demonstrated in an experimental setting of capsaicin-induced secondary hyperalgesia in humans [31] and in rodent models of trauma- or chemotherapy-induced neuropathic pain [1; 5; 29; 37; 46].

Animal studies of neuropathic pain have repeatedly demonstrated spinal sites of action for gabapentin in the modulation of evoked hypersensitivity [10; 11; 30; 47]. Consistent with this possibility, neuroimaging studies with capsaicin-induced sensitization in humans showed reduced BOLD signaling in brain regions processing nociceptive input from the spinal cord including the insula, the secondary somatosensory cortex (SII) and the brainstem, potentially reflecting gabapentin effects on spinal dorsal horn neurons [31; 64]. Alternatively, these results might be explained by direct gabapentin effects in the brain. Contributing to the uncertainty of how gabapentin may produce its effects, a recent clinical trial investigated the effects of continuous intrathecal gabapentin infusion through an implanted drug delivery system in chronic pain patients and surprisingly found no reduction in numerical pain rating scale or physical function, quality of life or emotional functioning [53]. Common side-effects of orally administered gabapentin include sedation, dizziness, and ataxia that are observed at doses used clinically for the treatment of neuropathic pain [64] suggesting that gabapentin produces at least some of its effects in the brain and that supraspinal activity could contribute to its analgesic efficacy. Our findings demonstrate that gabapentin has direct spinal actions on dorsal horn neurons in nerve injured rats likely accounting for antiallodynic actions observed. However, while intrathecal delivery is sufficient to alleviate aversiveness of ongoing nerve injury-induced pain, this requires activation of endogenous opioid signaling in the rACC. Additionally, rACC gabapentin is sufficient to relieve pain aversiveness with minimal effects on spinal neuronal responses to evoked stimuli and without reversing tactile allodynia. Collectively, these findings likely reflect direct actions of gabapentin in the rACC to modulate affective qualities of pain and spinal effects to modulate evoked hypersensitivity.

4.1 Effects of gabapentin in the spinal cord

Substantial evidence from animal studies suggests that after nerve injury, there is an up-regulation of $\alpha_2\delta$-1 in DRG neurons [13; 39; 62] and in the spinal cord [3; 6] that may contribute to neuronal hyperexcitability and spinal sensitization. Gabapentin binding to the $\alpha_2\delta$-1 subunit on presynaptic terminals of primary nociceptors in the dorsal horn of the spinal cord has been considered the key mechanism of anti-allodynic and anti-hyperalgesic effects (see [49] for review). However, studies in cultured DRG neurons demonstrate that gabapentin requires more than 17-20 hours to inhibit calcium current through binding to the $\alpha_2\delta$-1 subunit where neurotransmitter release may be influenced [26]. In contrast, *in vivo* behavioral and electrophysiological studies have shown that intrathecal gabapentin elicits effects within minutes [10; 11; 30]. Therefore the molecular mechanism of spinal effects remain unclear.

Consistent with these previous observations, our data demonstrate that in anesthetized SNL rats spinal application of gabapentin inhibits evoked neuronal discharge of spinal wide dynamic range neurons with effects observed typically within 10 minutes following

application, and a maximal inhibition typically observed by 30 minutes. Spinal gabapentin significantly suppressed the WDR neuronal activity evoked by noxious mechanical and thermal stimuli, as well as by electrical stimulation of Aδ- and C-fibers, input and brush. Likewise, intrathecal gabapentin time-dependently reversed both mechanical and thermal hypersensitivity in SNL rats, without producing changes in response thresholds in sham-operated rats, supporting direct inhibition of WDR neurons in the anti-hyperalgesic and anti-allodynic effects. Intrathecal gabapentin also elicited CPP and dopamine release in the NAc specifically in SNL rats, reflecting relief of aversiveness associated with ongoing pain. These anti-aversive effects of spinally administered gabapentin are ultimately mediated through supraspinal circuits, however, as both CPP and NAc DA release were abolished by blockade of opioid signaling in the rACC.

4.2 Supraspinal effects of gabapentin

The $\alpha_2\delta$-1 is also expressed in supraspinal sites including the areas involved with pain processing such as the cingulate cortex, amygdala, and the brainstem [12; 58] (Allen brain atlas). Gabapentin is an anticonvulsant and at analgesic doses readily crosses the blood brain barrier in both humans and rodents [4; 36]. Effects of systemic gabapentin on BOLD activity have been demonstrated using phMRI in naïve and SNL rats [22; 27]. Importantly, neuroimaging studies in healthy human subjects have demonstrated reduced mechanical stimulus-mediated BOLD fMRI activity during the oral gabapentin session in the insular cortex, the ACC and the SII [31]. The ACC, along with the thalamus and the brainstem, were also regions with increased activity during capsaicin-induced central sensitization. In a mouse model of neuropathic pain, intracerebroventricular gabapentin or pregabalin increased spinal norepinephrine levels and reduced mechanical and thermal hypersensitivity, suggesting activation of descending pain inhibitory pathways from locus coeruleus [57]. Whether gabapentin can alleviate affective features of ongoing pain by directly modulating the activity in these central circuits has not been determined.

Previously, we have reported that endogenous opioid activity within the rACC is required for CPP and NAc dopamine release following non-opioid, non-addictive treatments such as peripheral nerve block or spinal clonidine in rats with incisional or neuropathic pain, respectively [43]. Consistent with these findings, blockade of mu opioid receptors in the rACC with β-FNA blocks i.v. gabapentin-induced CPP and NAc DA release in SNL rats, demonstrating that opioid signaling in the rACC may represent a general mechanism necessary for relief of pain aversiveness. Also in agreement with our previous findings [43], pretreatment of rACC with β-FNA had no influence on the anti-allodynic actions of i.v. gabapentin, implying that the anti-allodynic effects are likely mediated at the spinal level and are partially separate from the affective aspects of pain.

Direct microinjection of gabapentin (100 μg) into the rACC was sufficient to produce CPP selectively in rats with neuropathic pain. Intra-rACC gabapentin also resulted in increased dopamine release in the NAc selectively in SNL but not sham rats, indicating that gabapentin can act in this brain region to relieve pain-induced aversiveness and facilitate pain relief-motivated behavior. In contrast, rACC gabapentin had no effect on nerve injury-induced mechanical allodynia. In spinal cord electrophysiological studies, rACC gabapentin

had no effect on noxious mechanical and thermal responses of WDR neurons. However, rACC gabapentin produced a small, but significant, inhibitory effect on electrically evoked excitability of spinal neuronal responses for C-fiber activity and post discharge. This indicates that rACC gabapentin might inhibit afferent nociception at the spinal cord level by engaging the descending pain modulatory pathways, although in our study, this effect was not sufficient to attenuate mechanical allodynia. Imaging studies in humans demonstrate that emotions and placebo analgesia can activate circuitry from the anterior cingulate cortex to the periaqueductal grey area in the brainstem to modulate unpleasantness of pain [9; 16; 60]. The same circuitry may therefore be involved in the observed effects on WDR neurons. It is noteworthy that gabapentin (100 μg) injected i.c.v. in mice with neuropathic pain was able to engage the descending pain inhibitory pathways directly from the brainstem [57]. Likewise, in subjects with capsaicin-induced hyperalgesia, oral gabapentin attenuated stimulus evoked neural activity in the brainstem descending pain modulatory regions [31; 64]. Systemic pregabalin in SNL rats attenuates mechanical evoked activity but not ongoing activity of thalamic neurons, part of the pathways that comprise the sensory components of pain [50]. Thus it is possible that whereas these agents can attenuate the ongoing aversive state produced by neuropathy as we show in the present study, they may not alter the ongoing nociceptive input.

It is important to point out that pharmacokinetics of gabapentin in humans and rodents is different [61], which may explain higher systemic and intrathecal doses used in this and other rodent studies [17; 20; 23; 24; 38; 68]. For intra-rACC injections, we used 10 and 100 μg in anesthetized and awake rats, respectively. Following oral dosing of 200 mg/kg in rats, a PK/PD study observed maximum concentration of gabapentin in the brain extracellular fluid at approximately 10 μg/ml [61]. Thus, CNS tissue concentrations of GBP following intra-rACC injections, are likely higher than those achieved following systemic dosing in rats. Concentrations that are achieved clinically in specific brain regions relevant to the actions of gabapentin remain unknown.

In conclusion, this study provides evidence that gabapentin can act directly in the spinal cord to suppress nociceptive responses of spinal cord neurons and attenuate nerve injury-induced hypersensitivity. As the affective qualities of pain are closely linked to intensity of nociceptive inputs, spinal actions of gabapentin were also shown to be sufficient to reduce pain aversiveness. Ultimately, however, pain relief-motivated behavior, but not evoked hypersensitivity, requires engagement of endogenous supraspinal circuits including opioid signaling in the rACC and dopamine signaling in the NAc. Gabapentin may also act directly in the rACC to modulate pain without significantly changing evoked hypersensitivity. Whether rACC gabapentin can modulate descending pain pathways to inhibit nociceptive traffic at the spinal cord requires further study, but preclinical investigations have shown that gabapentin can act in the brainstem to engage descending pain modulatory pathways. Thus, the clinical efficacy of gabapentin could reflect the outcome of congruent effects at spinal and supraspinal sites that modulate different aspects of pain. Reduction of pain is ultimately the main goal of pain therapy and it is likely that in patients with neuropathic pain, supraspinal actions preferentially contribute to gabapentin’s analgesic effects.

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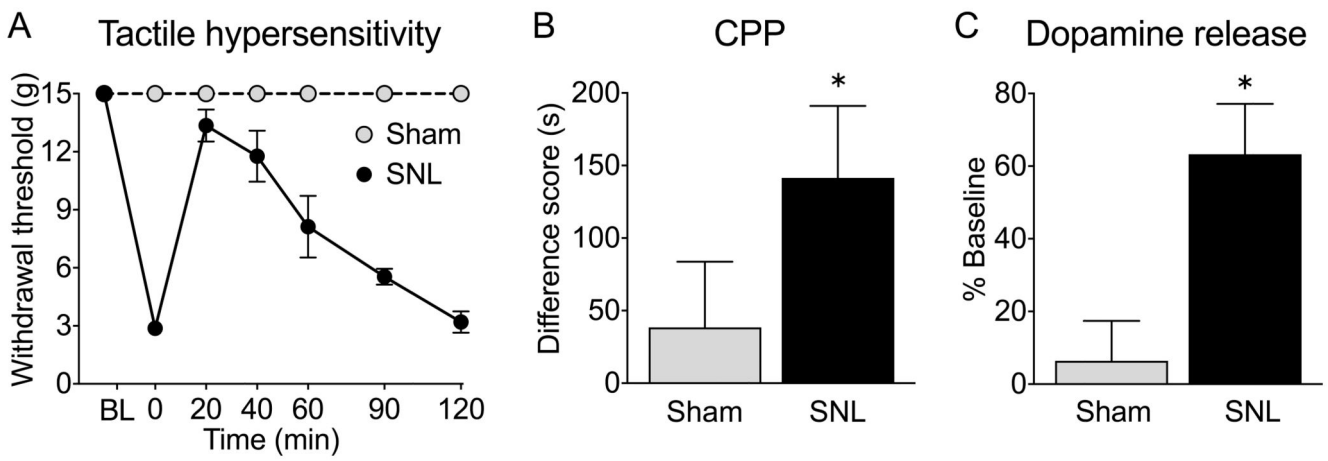

Figure 1. Effects of intravenous gabapentin on tactile allodynia, CPP and NAc dopamine release in SNL rats.

(A) Intravenous administration of gabapentin (50 mg/kg) temporarily reversed nerve injury-induced tactile allodynia in SNL rats (sham: n=7; SNL: n=9;). (B) Rats with neuropathic pain but not sham operated animals demonstrated preference for the gabapentin-paired chamber as shown by significantly increased difference score, suggesting that i.v. gabapentin relieves ongoing neuropathic pain (sham: n=10; SNL: n=17; *$p < 0.05$ compared with pre-conditioning time spent in chamber). (C) I.v. gabapentin increased DA efflux in the NAc shell of SNL but not sham-operated animals demonstrating rewarding effects of pain relief (sham: n=11; SNL: n=12; *p < 0.05 compared to the sham group). Data are means ±SEMs.

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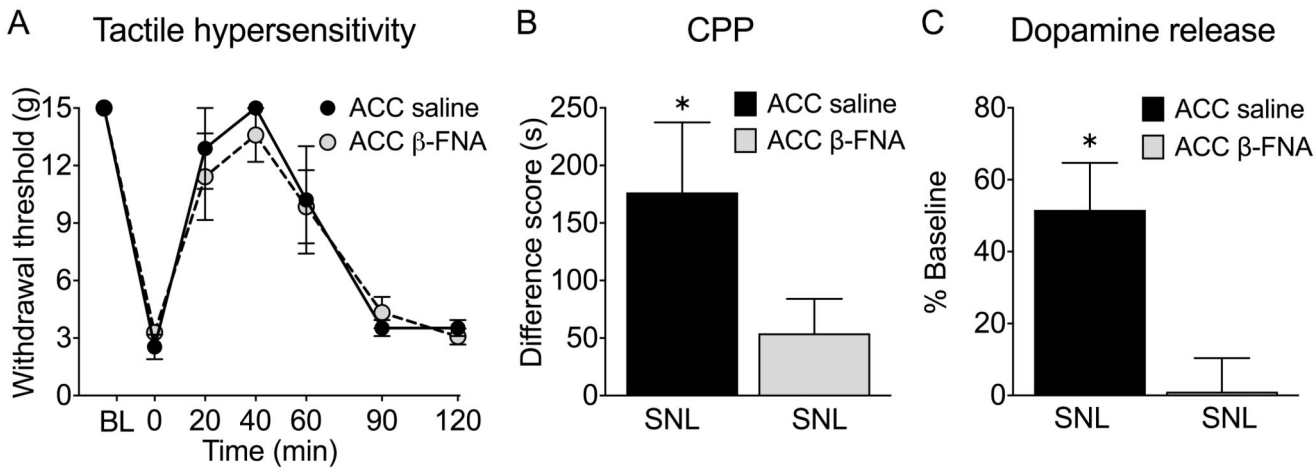

Figure 2. Pretreatment with β-FNA into the rACC of SNL rats blocks intravenous gabapentin induced CPP and dopamine efflux in NAc while the anti-allodynic effects are preserved.

(A) SNL rats were pretreated 20-24 h before testing with irreversible opioid receptor antagonist β-FNA (3 μg) or vehicle (saline) into the rACC. The efficacy and time course of anti-allodynic effects of i.v. gabapentin were statistically indistinguishable in both groups (saline: n=4; β-FNA: n=6). (B) In contrast, rACC β-FNA reduced the ability of i.v. gabapentin to elicit CPP in SNL rats (saline: n=7; β-FNA: n=12; $*p < 0.05$ compared with pre-conditioning time spent in chamber). (C) Pretreatment with rACC β-FNA eliminated dopamine release in response to i.v. gabapentin (saline: n=8; β-FNA: n=9; *p < 0.05). Data are means ±SEMs.

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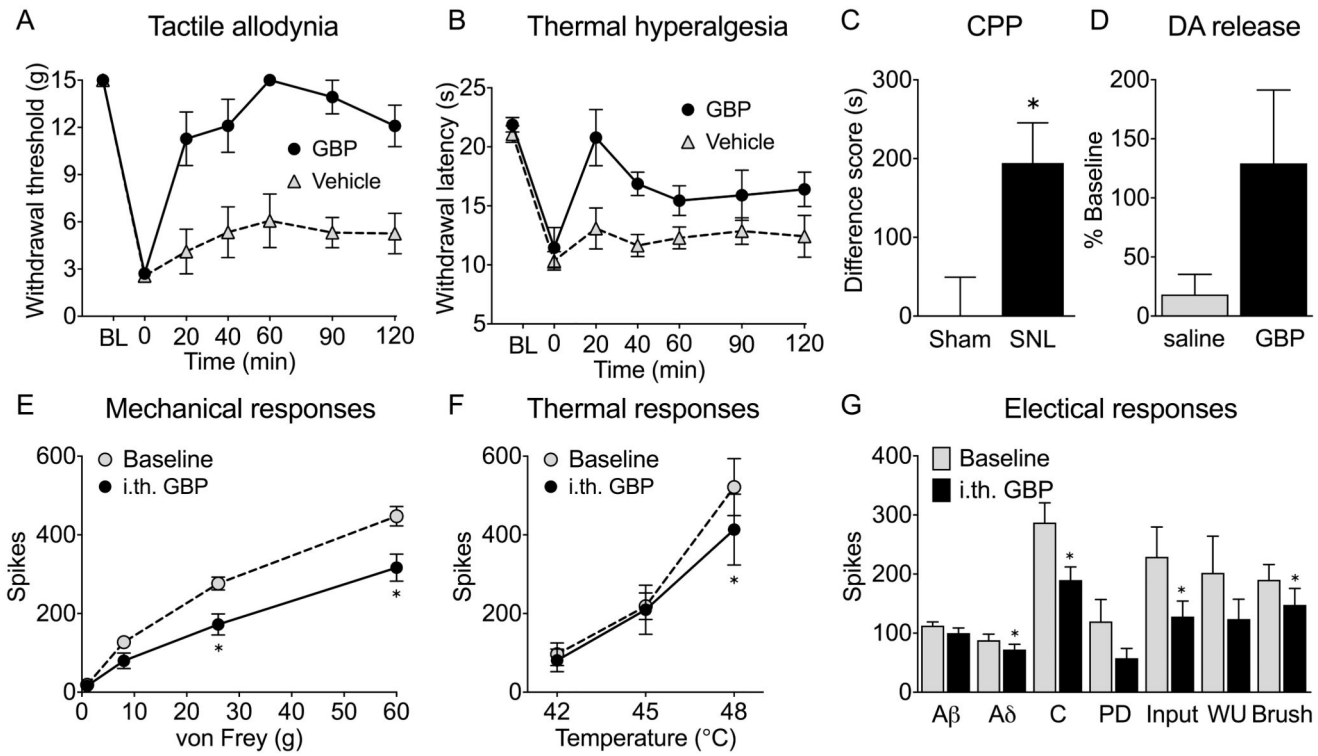

Figure 3. Effects of intrathecal gabapentin on pain thresholds, CPP, NAc dopamine release and the activity of WDR neurons in SNL rats.

SNL surgeries produced (A) tactile allodynia and (B) thermal hyperalgesia in rats when tested 14 days following spinal nerve ligation. Intrathecal administration of gabapentin (200 μg) blocked SNL-induced tactile allodynia and thermal hyperalgesia (saline: n= 9; GBP: n= 10). (C) Rats with SNL demonstrated significant increase in the time spent in the i.th. gabapentin-paired chamber while sham-operated rats showed no preference (sham: n= 10; SNL: n= 12; *$p < 0.05$ compared with pre-conditioning time spent in chamber). (D) In SNL rats i.th. gabapentin but not i.th. saline elicited dopamine efflux in NAc shell (saline: n=7; GBP: n=8). (E) In SNL rats, spinal application of gabapentin inhibited responses of WDR neurons to noxious mechanical stimulation with von Frey filaments of increasing strength (n=5; *p < 0.05 compared to pre-drug baseline). Spinal application of gabapentin inhibited responses of WDR neurons to noxious (48°C) heat (n=5; *p < 0.05 compared to pre-drug baseline). (F) After SNL, significant inhibitory effects of spinal gabapentin were observed for Aδ-fiber, C-fiber, Input and Brush responses (n=5; *p < 0.05 compared to pre-drug baseline). Data are means ±SEMs.

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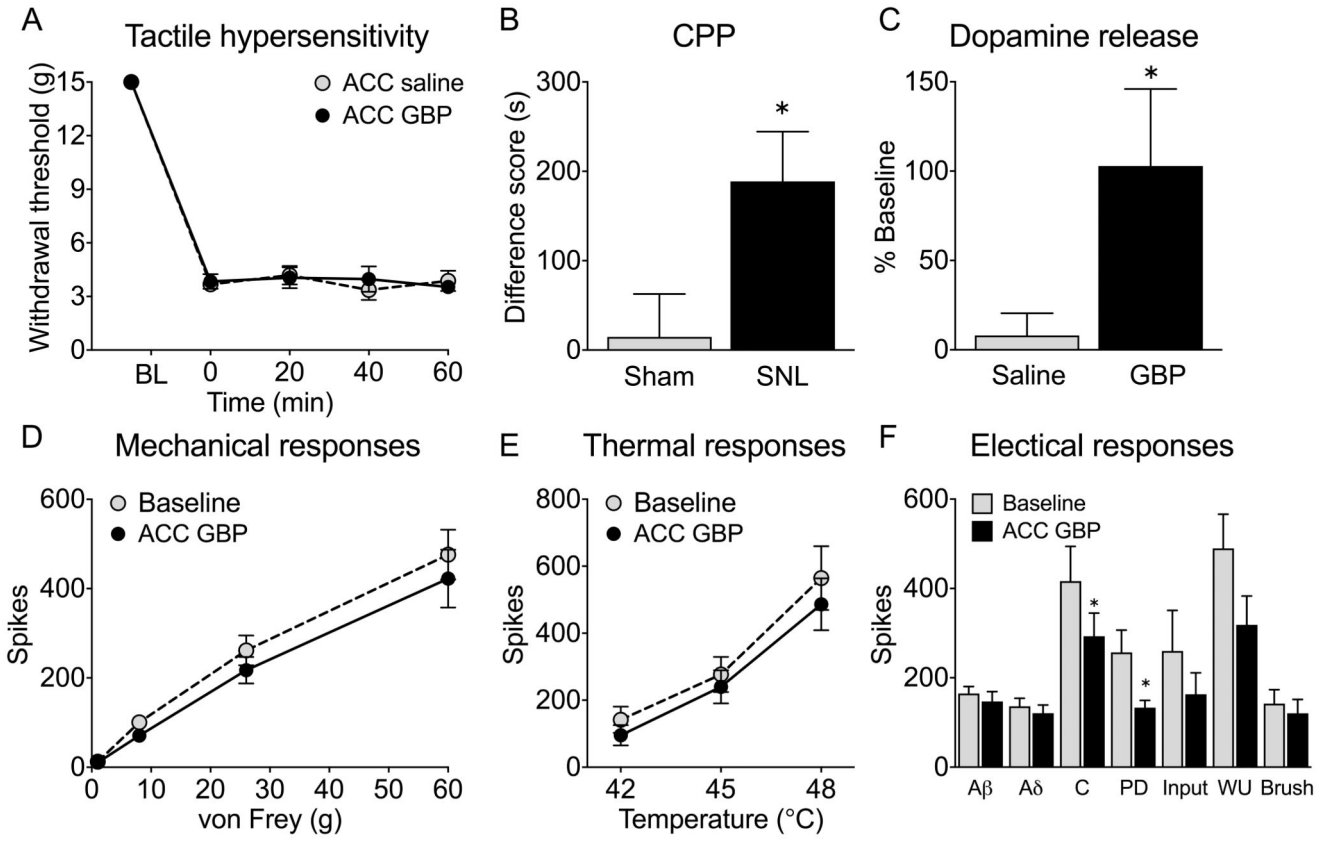

Figure 4. Effects of gabapentin administration into the rACC on tactile allodynia, CPP, NAc dopamine release and the activity of WDR neurons in SNL rats.

(A) Bilateral administration of gabapentin into the rACC (100 μg) did not reverse SNL-induced tactile hypersensitivity (n=5 in each group). (B) In contrast, rACC gabapentin produced CPP selectively in SNL but not sham rats (n=8 in each group; *$p < 0.05$ compared with pre-conditioning time spent in chamber). (C) Local injection of gabapentin into the rACC also increased the levels of extracellular dopamine in the NAc only in SNL rats (saline: n=8; GBP: n=5; *p < 0.05). (D) In SNL rats, rACC administration of gabapentin had no effect on the responses of WDR neurons to noxious mechanical stimulation (n=5). (E) Gabapentin injections into the rACC had no effect on the responses of WDR neurons to heat stimulation (n=5). (F) Significant inhibitory effects of rACC gabapentin were observed for C-fiber and Post Discharge responses (n=5; *p < 0.05 compared to pre-drug baseline). Data are means ±SEMs.