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Gina LC Yosten, … , Willis K. Samson, Daniela Salvemini

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GPR160 de-orphanization reveals critical roles in neuropathic pain in rodents

Gina Yosten[1,2], Caron M. Harada[1,2], Chris Haddock[1,2], Luigino Antonio Giancotti[1,2], Grant R. Kolar[2,3], Ryan Patel[4], Chun Guo[1], Zhoumou Chen[1,2], Jinsong Zhang[1,2], Timothy M. Doyle[1,2], Anthony H. Dickenson[4], Willis K. Samson[1,2] and Daniela Salvemini[1,2*]

[1]Department of Pharmacology and Physiology, [2]Henry and Amelia Nasrallah Center for Neuroscience and [3]Department of Pathology Saint Louis University School of Medicine, St. Louis, MO 63104 USA and [4]Neuroscience, Physiology and Pharmacology University College London, Gower St. London WC1E 6BT

*Daniela Salvemini: E-mail:daniela.salvemini@health.slu.edu, ORCID ID: 0000-0002-0612-4448, Phone: 1-314-977-6430, Fax: 1-314-977-6441, Address: 1402 South Grand Blvd, St. Louis, MO 63104, USA

Conflict of Interest: Drs. Salvemini, Yosten and Samson have a patent on the discovery (WO2017011738). All other authors claim no conflicts of interest.

Abstract

Treating neuropathic pain is challenging and novel non-opioid based medicines are needed. Using unbiased receptomics, transcriptomic analyses, immunofluorescence and in situ hybridization, we found the expression of the orphan GPCR (oGPCR) *Gpr160* and GPR160 increased in the rodent dorsal horn of the spinal cord (DH-SC) following traumatic nerve injury. Genetic and immunopharmacological approaches demonstrated that GPR160 inhibition in the spinal cord prevented and reversed neuropathic pain in male and female rodents without altering normal pain response. GPR160 inhibition in the spinal cord attenuated sensory processing in the thalamus, a key relay in the sensory discriminative pathways of pain. We also identified cocaine- and amphetamine-regulated transcript peptide (CARTp) as a GPR160 ligand. Inhibiting endogenous CARTp signaling in spinal cord attenuated neuropathic pain, whereas exogenous intrathecal (i.th.) CARTp evoked painful hypersensitivity through GPR160-dependent ERK and cAMP response element-binding protein (CREB). Our findings de-orphanize GPR160, identify it as a determinant of neuropathic pain and potential therapeutic target, and provide insights to its signaling pathways. CARTp is involved in many diseases including depression, reward and addiction, de-orphanization of GPR160 is a major step forward understanding the role of CARTp signaling in health and disease.

Introduction

Neuropathic pain conditions arising from injuries to the nervous system due to trauma, disease or neurotoxins are chronic, severe, debilitating and exceedingly difficult to treat (1). Opioids are widely used to treat chronic pain but limited by severe side effects and strong abuse liability (2). Neuropathic pain is a burgeoning global medical issue (e.g., >15 million people in the US (3) and >20% of the European population (4)) with a profound annual economic burden of treatment (5). When combined with over 15 million people worldwide having experienced an opioid use disorder (6), a high priority has been placed upon developing novel non-opioid based analgesics.

GPCRs are the most abundant receptor family and regulate a diverse array of cellular functions, including neurotransmission in pain (7). Approximately 120 of 400 non-sensory GPCRs are considered oGPCRs as their cognate ligands are unknown (8). Using a multidisciplinary approach, we present the first evidence that the oGPCR GPR160 in the spinal cord plays critical roles in the development and maintenance of hypersensitivity associated with traumatic nerve injury-induced neuropathic pain. We also identify CARTp (9) as a GPR160 ligand and unraveled signaling pathways engaged downstream of GPR160. These findings provide the foundation for investigating GPR160 as a potential therapeutic target for treating chronic pain.

Results and Discussion

GPR160 is upregulated in the spinal cord during neuropathic pain

Our receptomic approach (10) (Supplemental Figure 1A) identified 4 main clusters (Supplemental Figure 1B) among non-orphan GPCRs known to be altered in neuropathic pain states (11, 12). Querying the sequence homology of oGPCRs against GPCRs in these clusters identified 31 candidate oGPCRs (Supplemental Figure 1C). We surveyed their expression in the spinal cord from rats with chronic constriction of the sciatic nerve (CCI)-induced neuropathic pain, which produces mechano-hypersensitivities that peak by d7 and lasts for several weeks (13). PCR and quantitative PCR analyses of these oGPCRs revealed that *Gpr160* significantly increased in the dorsal, but not the ventral, horn of the spinal cord ipsilateral to injury (Figure 1A). As a control, *Gpr107*, which is expressed in the dorsal horn of the spinal cord but not homologous to any of our branch clusters, was not significantly changed by CCI (Figure 1A). No significant changes in *Gpr160* were observed in the dorsal root ganglia (n=3; p=0.5).

RNA-Seq analyses of ipsilateral rat DH-SC following CCI or SHAM injury identified 60 differential expressed GPCRs between CCI and SHAM groups. Remarkably, *Gpr160* was one of the *Gpr* transcripts with the greatest differential expression (4.44-fold change; false discovery rate = 6.06×10^{-12}) in the CCI group (Figures 1B,C).

GPR160 is highly conserved among species and expressed on neurons, astrocytes and microglia (14, 15) in human and rodent CNS, including the spinal cord (15, 16). Image analyses of spinal cord from rats with CCI revealed increased GPR160 (26.9%±5.6%SEM; n=5/group; p=0.042; paired *t*-test; Figure 1D) and *Gpr160* (Figure 1E) within lamina 1 and 2 of ipsilateral DH-SC compared to the contralateral side. Although

found in astrocytes (Supplemental Figures 2A and 3B), microglia (Figure 1F; Supplemental Figure 3C) and neurons (Supplemental Figures 2C and 3D), *Gpr160* was significantly increased in proximity to *Aif1* (Figure 1G), but not *Gfap* (Supplemental Figure 2B) or *Rbfox3* (Supplemental Figure 2D); suggesting microglia may account for CCI-induced *Gpr160* and GPR160 expression in the spinal cord.

Activation of GPR160 in the spinal cord contributes to neuropathic pain

The functional contribution of GPR160 at this site was tested using genetic (siRNA) and immunopharmacological (neutralizing antibody) approaches, since there are no small molecule GPR160 antagonists. Daily i.th. injections of si*Gpr160*, but not control siRNA (si*Gfp*), blocked mechano-allodynia in the rat CCI model (Figure 2A) and a second rat model of traumatic nerve injury (spared nerve injury, SNI (17); Figure 2B). Moreover, i.th. si*Gpr160* at a time of peak CCI-induced mechano-allodynia (d7 and d8) significantly reversed allodynia (Figure 2C) and reduced *Gpr160* in the ipsilateral DH-SC by approximately 40% (n=5). Intrathecal injection of neutralizing GPR160 antibody (GPR160 ab) at a time of peak neuropathic pain (d8) also reversed mechano- and cold-allodynia in male rats by 30min with peak reversal effects by 2h (shown in Figures 2D,E) and resolution by 6h. Similar results were obtained in female rats (Figure 2F). GPR160 inhibition did not produce observable adverse health effects or alter normal nociceptive thresholds (tail flick latency (18)) in non-injured rats (Figure 2G). These results suggest selective alleviation of chronic pain states without impact on beneficial and protective nociceptive responses.

We examined the effect of GPR160 ab on neuronal processing within the spinothalamic-ventrobasal-somatosensory cortical pathway to peripherally applied sensory modalities using in vivo electrophysiological recordings from the ventral posterolateral thalamus (Supplemental Figure 4A), a key relay in the sensory discriminative pathways of pain in the brain. Baseline evoked and ongoing neuronal activities were comparable to our previous observations (19). Intrathecal GPR160 ab produced modality-selective inhibitory effects similar to pregabalin (19) in rats with spinal nerve ligation (SNL) that were dependent on the pathophysiological state; similar to behavior and demonstrative of sensory processing rather than motor responses. When compared to baseline, the neuronal responses to punctate mechanical stimuli following GPR160 ab were reduced to a range of low intensity von Frey filaments (2 and 8 g) and those likely to exceed withdrawal thresholds (>15g); whereas, there were no changes observed in sham groups (Figure 2J and Supplemental Figure 4B). No inhibitory effects were observed on evoked neuronal responses to heat (Supplemental Figure 4C), innocuous (Supplemental Figure 4D) and noxious evaporative cooling (Supplemental Figure 4E) or brush stimulation of the receptive field (Supplemental Figure 4F) in either group. Ongoing neuronal activity was also unaltered post-dosing in either group (Figure 2K). A control antibody (IgG) in naïve rats had no effect on all evoked and spontaneous measures (Figures 2L,M and Supplemental Figure 4). These results show that GPR160 in spinal cord contributes to the ascending transmission of sensory inputs within sensory-discriminative projection pathways. The lack of effect in sham animals and on thermal and ongoing activity indicates a selective role of GPR160 inhibition in these pathophysiological evoked responses that is similar to pregabalin (19). One discrepancy

between the behavioral and neuronal data was the lack of effect in cold responses of the sensory neurons. This may be due to the complexities of cold processing and the suprathreshold nature of the neuronal cold stimulus that might employ different molecular mechanisms between behavioral and electrophysiological tests. Differences between the models used may also contribute. The neuronal responses extend to suprathreshold stimuli and so represent coding of high intensity stimuli that could equate better to high pain scores in patients with neuropathy than threshold responses that can be measured in behavioral studies (20).

CARTp - a ligand of GPR160

Comparing tissue expression profiles (NCBI Gene) of GPR160 and endogenously expressed orphan ligands revealed high correlation between GPR160 and CARTp. CARTp has two bioactive forms in rat [CARTp (55–102) and CARTp (62–102) (21)] and human [CARTp (42–89) and CARTp (49–89) (21)]. CARTp (55-102), the most widely used isoform, acts through a $G\alpha_{i/o}$-coupled GPCR linked to the activation (phosphorylation) of ERK (22, 23). Using cell culture, we de-orphanized GPR160 by identifying a functional and potential physical connection between CARTp and GPR160. In human KATOIII cancer cells that express GPR160 (Supplemental Figure 5A), CARTp induced *cFOS* expression, which was completely blocked in si*Gpr160*-transfected cells (Figure 3A). Rat pheochromocytoma cells (PC-12), which can be differentiated into a neuronal-like phenotype, expressed GPR160 (Supplemental Figures 5B,C). CARTp stimulated ERK phosphorylation (pERK) in PC-12 cells that was attenuated in si*Gpr160*-transfected cells (Figure 3B). The efficacy of siRNA was confirmed by reduced *Gpr160*

and GPR160 (Supplemental Figures 5B,C). These findings suggest a functional relationship between CARTp and GPR160 and reveals GPR160 confers CARTp-induced *cFOS* and phosphorylation of ERK. Moreover, CARTp co-immunoprecipitated with GPR160 (Figure 3C), suggesting a potential physical interaction. The 75 kDa complex is greater than the predicted 52 kDa complex and may reflect additional proteins that associate to form the signalosome. We also found exogenous CARTp co-localized to GPR160 (Figure 3D-F). We have previously demonstrated that GPR160 does not interact with another peptide hormone, proinsulin C-peptide (24); suggesting the potential CARTp-GPR160 interaction is likely specific.

CARTp is expressed in the CNS, including the superficial laminae of the rat spinal cord (25, 26). *Cartp* is expressed in mouse cerebral cortex glia (microglia and astrocytes) and neurons (14). However, the contribution of CARTp/GPR160 signaling to nociceptive processing is not known. An i.th. injection of a neutralizing CARTp antibody (CARTp ab) at a time of peak CCI-induced neuropathic pain reversed mechano-allodynia in mice and rats in a time-dependent fashion by 30min with peak reversal by 2h (Figures 2H,I) and resolution within 5 to 6h. The effects of CARTp ab mimicked those noted with GPR160 inhibition (Figures 2D,E); suggesting that CARTp/GPR160 signaling occurs in response to nerve injury.

CARTp induces GPR160-mediated hypersensitivities through ERK/CREB signaling in the spinal cord

If CARTp/GPR160 in the spinal cord following nerve injury contributes to the development of neuropathic pain, then i.th. injections of CARTp should recapitulate

behavioral consequences of neuropathic pain states. Results from early studies of CARTp in pain were inconclusive due in part to limited data and contradictory pro- and anti-nociceptive effects in the CNS (25, 27, 28). Reported antinociceptive effects of CARTp manifested at very high doses of the peptide (µg) and the purity of the peptide preparation was not known or not reported (27, 28). In contrast, Ohsawa and colleagues reported that i.th. injections of low dose (3-100 ng), highly purified (>96%) CARTp induced thermal hyperalgesia in a dose-dependent fashion (25). Using the same purity of CARTp and supplier as Ohsawa, a single i.th. injection of CARTp (3-30ng) in mice caused profound mechano-allodynia in pilot studies that peaked by 1h and persisted over 5h. As shown in Figure 4A, CARTp (30 ng) caused near-to-maximal allodynia by 1h that was abolished by i.th. injection of GPR160 ab (Figures 4A and B) or CARTp ab (Figure 4C), providing support that CARTp-induced mechano-hypersensitivity is dependent on GPR160.

CARTp-induced ERK signaling stimulates the phosphorylation and activation of CREB independently of cAMP signaling (22). ERK can serve as an upstream regulator of CREB phosphorylation (pCREB) (29) during the development of neuropathic pain (30). We found i.th. CARTp induced GPR160-mediated ERK/CREB signaling in the mouse DH-SC that contributed to the development of mechano-allodynia. First, i.th. CARTp induced the phosphorylation of ERK (Figure 4D) and CREB (Figure 4E), which was attenuated by co-administration of GPR160 antibody (Figures 4D,E). Secondly, inhibiting MAPK/ERK kinases (MEK) 1 and 2 with the MEK1/2 inhibitor, U0126 (31), attenuated CARTp-induced mechano-allodynia (Figure 4F) and phosphorylation of ERK (Figure 4D) and CREB (Figure 4E). Finally, CARTp-induced mechano-allodynia (Figure 4F) and CREB

phosphorylation (Figure 4E) were attenuated by i.th. administration of the CREB inhibitor, 666-15 (32).

Using an array of multidisciplinary approaches, we found a role for CARTp/GPR160 signaling (Figure 4G) in spinal cord in neuropathic pain. These findings provide the kernel for future investigation of GPR160 signaling in pain and other CARTp-associated diseases, including anxiety and depression, reward and addiction, food intake and maintenance of body weight (21). Accordingly, our findings set the stage for medicinal discovery efforts to identify small molecule antagonists of GPR160 for the treatment of neuropathic pain with broader implication for the treatment of additional disease states.

Methods

Detailed methods are provided in the Supplemental Materials.

Study Approval. All experiments were performed in accordance with the International Association for the Study of Pain, the National Institutes of Health guidelines on laboratory animal welfare, The Animals (Scientific Procedures) Act 1986/directive 2010/63/EU and approved by the Saint Louis University Institutional Animal Care, internal ethics committee at the University College London and the UK Home Office.

RNA-seq data are available through the GEO repository (#GSE143895).

Author contributions: Experiments and data analyses: GY, CMH, CH, LAG, GK, CG, ZC, JZ, TMD, RP, A.D. Assisted in writing manuscript: CMH, LAG, GK, JZ, TMD, R.P, A.D. Conceived experiments and wrote the manuscript: GY, WKS, DS.

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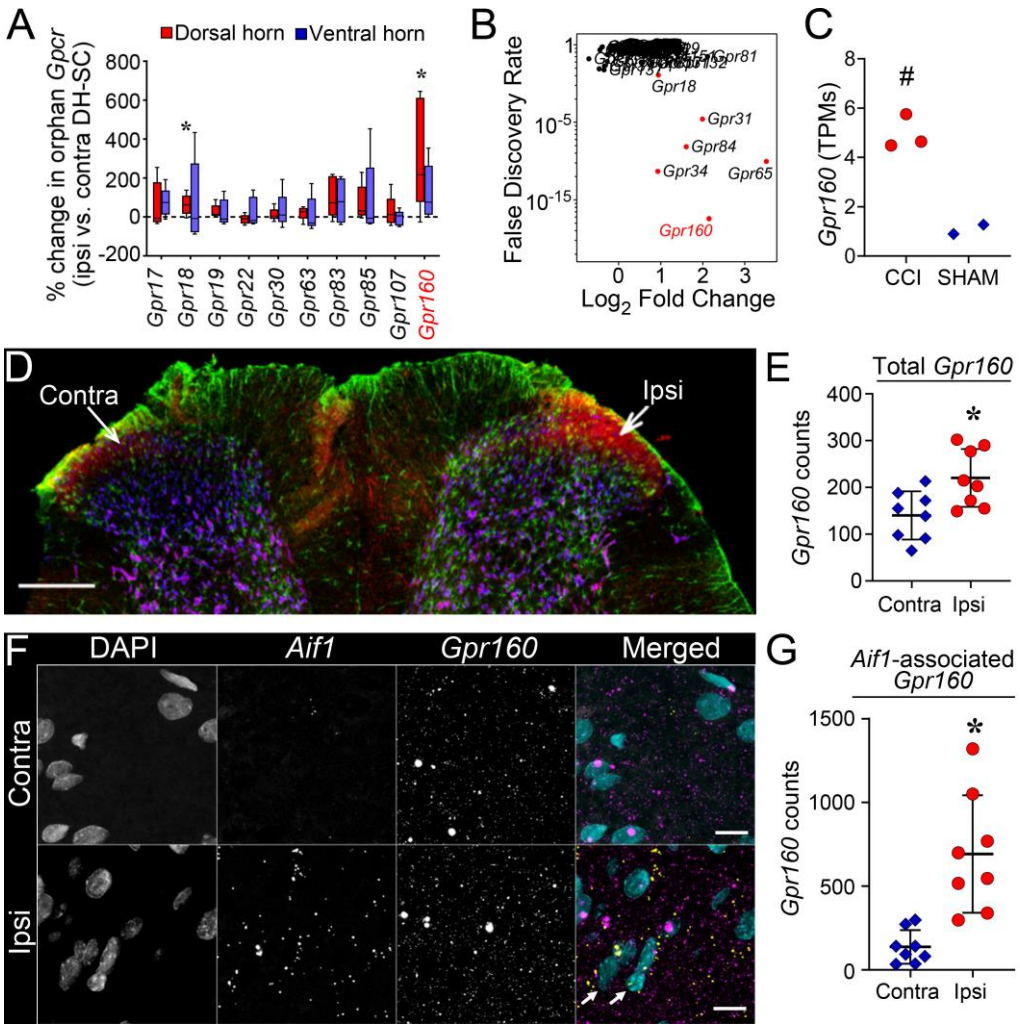

Figure 1. *Gpr160* and GPR160 upregulation in the spinal cord following CCI. (**A**) Quantitative real-time PCR analysis of oGPCR mRNA expression in the dorsal and ventral horns of the spinal cord from rats with CCI on d7 (n=5). (**B,C**) RNA-Seq analyses of rat DH-SC ipsilateral to CCI on d9: (**B**) Differential expression of 60 GPCRs between CCI and sham (n=3/group). (**C**) *Gpr160* in CCI and SHAM. TPMs=total reads per million. (**D**) Immunolabeled GPR160 (red) in lamina I/II spinal cord of rats with CCI. Ipsilateral (Ipsi), contralateral (Contra), GFAP (green) and NeuN (blue). (**E-G**) RNA-scope analyses of the rat DH-SC on d10 post CCI: (**E**) Quantitation of total *Gpr160*. (**F,G**) Association (white arrows; **F**) of *Gpr160* (magenta) and *Aif1* (microglia; yellow) increased ipsilateral to CCI (**G**). DAPI (cyan). Scale bar = 100 μm (**D**) or 10 μm (**F**). Data are expressed as (**A**) median, interquartile range and minimum/maximum values or (**E,G**) mean±SD. (**A-C,E,G**) Data analyzed by two-tailed Student's *t*-test; (**B,C**) adjusted by Benjamini-Hochberg false discovery rate. *****P<0.05 versus Contra and #P<0.05 and q<0.05 versus SHAM.

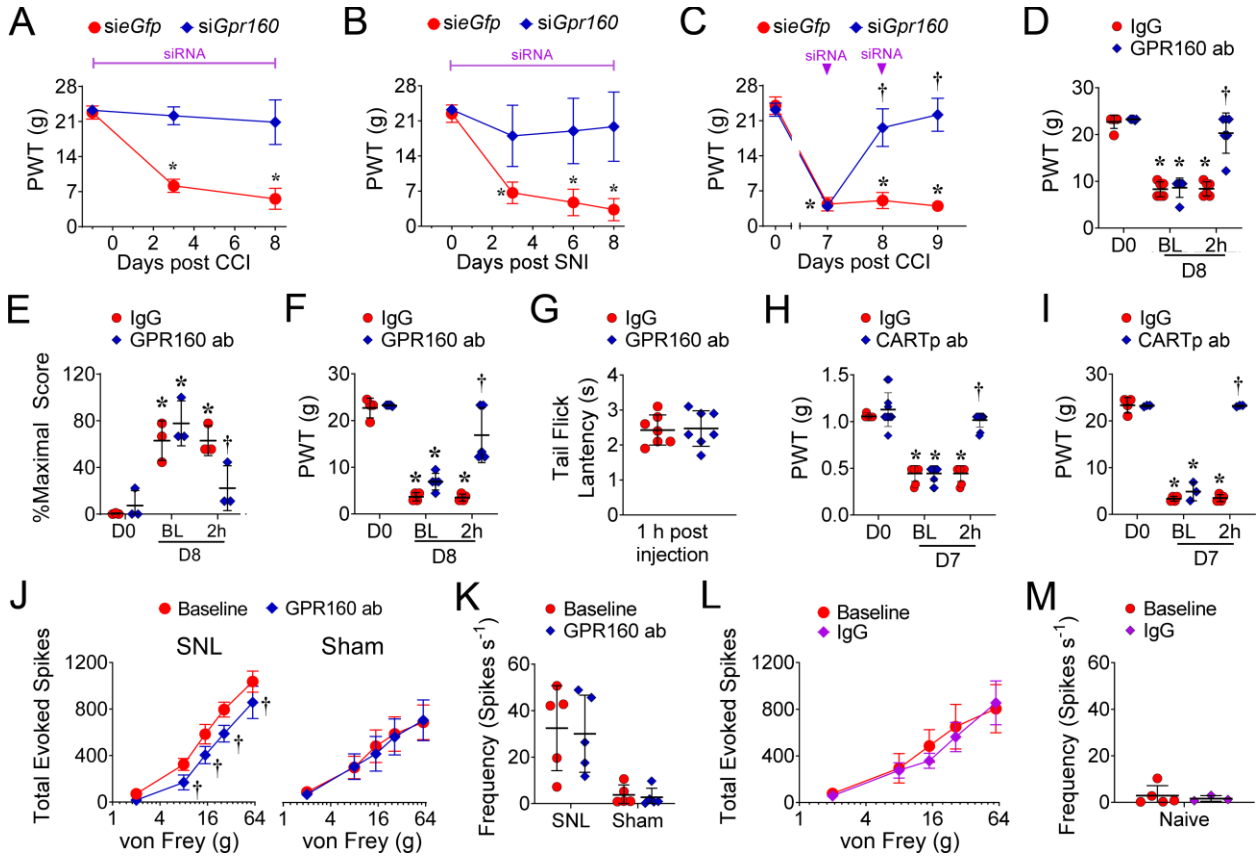

Figure 2. GPR160 inhibition attenuated and reversed neuropathic pain. (**A**) CCI- and (**B**) SNI-induced mechano-allodynia in male rats were prevented by daily i.th. si*Gpr160* (**A:** n=6; **B:** n=4), but not si*eGfp* control (**A:** n=7; **B:** n=4). (**C**) CCI-induced mechano-allodynia in male rats was reversed by i.th. si*Gpr160*, but not si*eGfp* (n=3/group). CCI-induced (**D**) mechano- (n=6/group) and (**E**) cold-allodynia (n=3/group) in male rats was reversed with i.th. GPR160 ab, but not with non-specific IgG. (**F**) Intrathecal GPR160 ab (n=4), but not IgG (n=5), reversed CCI-induced mechano-allodynia in female rats. (**G**) Intrathecal GPR160 ab or IgG (n=7/group) in normal male rats had no effect on tail-flick nociceptive responses. (**H,I**) Intrathecal CARTp ab (**H**: n=11, **I**: n=3), but not IgG (IgG; **H:** n=8; **I:** n=4), reversed CCI-induced mechano-allodynia in male mice (**H**) and rats (**I**). When compared to baseline, i.th. GPR160 ab (n=5) attenuated neuronal responses to punctate mechanical stimuli (**J**), but not ongoing neuronal activity, (**K**) in SNL, but not sham, rats. No effects on neuronal responses to punctate mechanical stimuli (**L**) or ongoing neuronal activity (**M**) were observed with IgG (n=4) in naïve rats. Data are expressed as mean±SD (**A-I**) or mean±SEM (**J-M**) and analyzed by two-tailed, (**A-F,H-L**) two-way repeated measures ANOVA with Bonferroni comparisons and (**G,M**) *t*-test. *****P<0.05 versus d0 (D0) and †P<0.05 versus baseline (BL).

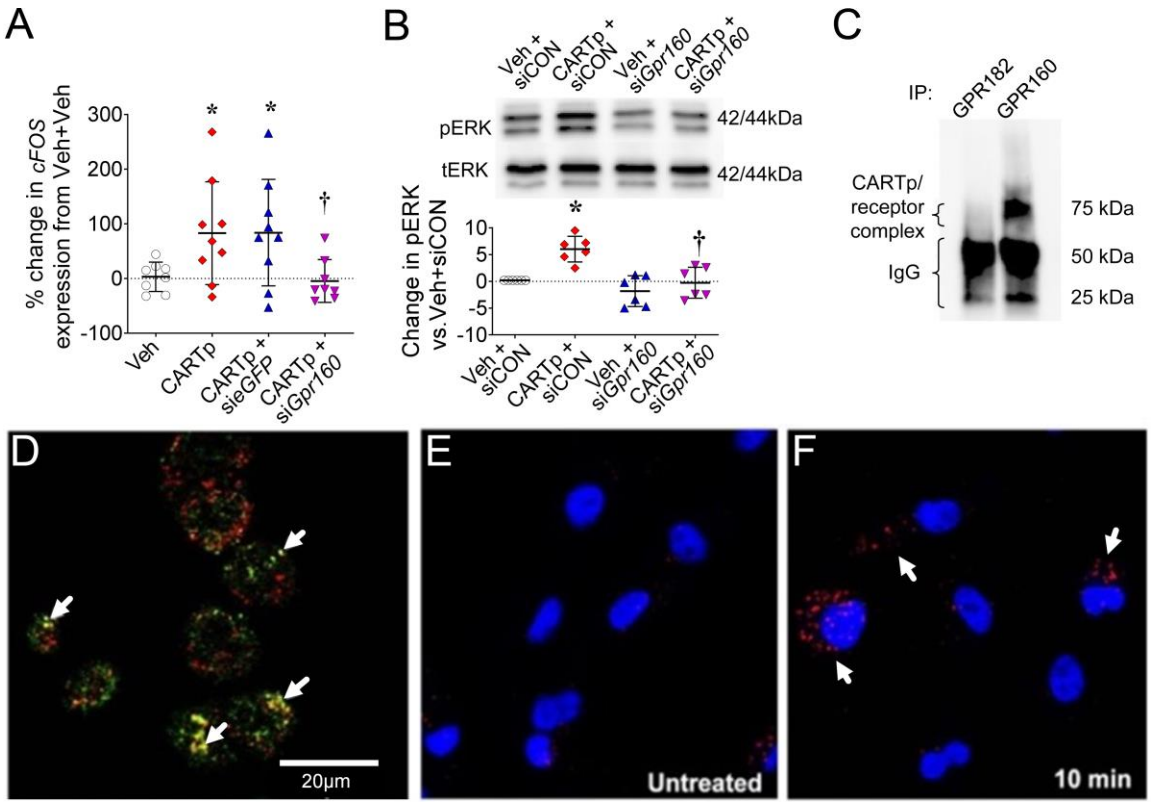

Figure 3. CARTp is a GPR160 ligand. (**A,B**) CARTp stimulated (**A**) *cFOS* in human KATOIII cells and (**B**) ERK phosphorylation in PC-12 cells; events attenuated with si*Gpr160*, but not control siRNA (**A**: si*eGfp* or **B**: siCON, non-coding scrambled siRNA; *n*=3 experiments/group with 3 replicates/experiment). tERK= total ERK (**C**) In KATOIII cell lysates, exogenous CARTp co-immunoprecipitated with GPR160 (*n*=3 experiments/group with 3 replicates/experiment). (**D**) FAM-labeled CARTp (*green*) colocalized (*yellow; white arrows*) with GPR160 (*red*) in KATO III cells (n=3 experiments with 1 replicate/experiment). (**E,F**) Proximity ligation assay revealed the close proximity (*red*) of CARTp and GPR160 (n=3 experiments with 2 replicates/experiment) in CARTp-treated cells (**F**), but not in untreated cells (**E**); blue = nuclear staining. Data are expressed as mean±SD and analyzed by two-tailed one-way ANOVA with Dunnett's comparisons. *****P<0.05 versus Veh and †P<0.05 versus CARTp + si*Gfp*/siCON.

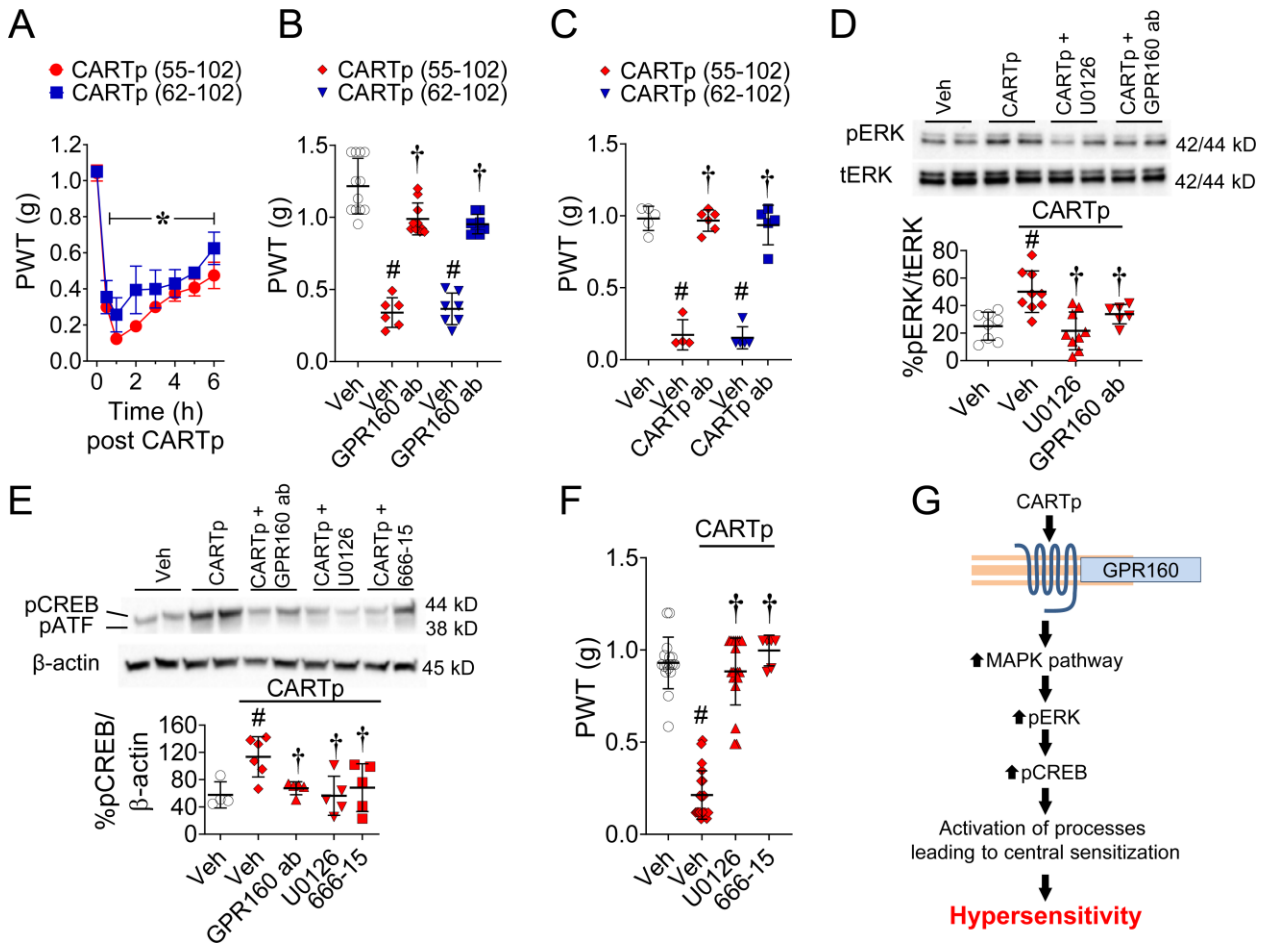

Figure 4. CARTp induced mechano-hypersensitivity in mice through GPR160-dependent ERK-CREB activation in the spinal cord. (**A**) Time-dependent development of mechano-allodynia in mice (n=4) after i.th. CARTp (55-102) or CARTp (62-102). (**B,C**) Mechano-allodynia measured at 1h after i.th. CARTp (55-102) (**B:** n=6; **C**: n=4) or CARTp (62-102) (**B:** n=7; **C:** n=5) was reduced with i.th. GPR160 ab (**B**; n=10 and n=8, respectively) or i.th. CARTp ab (**C**; n=6 and n=5, respectively). (**D,E**) When compared to vehicle (**D:** n=8, **E:** n=4), i.th. CARTp (55-102) induced phosphorylation of ERK (**D**; pERK; n=9) and CREB (**E**; pCREB, n=6) in the DH-SC, which was attenuated with i.th. co-injections of MEK inhibitor (U0126; **D:** n=9, **E:** n=5), CREB inhibitor (666-15; **e:** n=5) or GPR160 ab (**D:** n=6). (**F**) CARTp (55-102)-induced mechano-allodynia (n=20) was attenuated with co-injection of U0126 (n=20) or 666-15 (n=6). Vehicles to CARTp, U0126 and 666-15 (n=17) had no effect on behavior. (**G**) Proposed model of CARTp/GPR160-induced signaling. Data are expressed as mean±SD and analyzed by two-tailed (**A**) two-way ANOVA with Bonferroni comparisons or (**B-F**) one way ANOVA with Dunnett's comparisons. *****P<0.05 versus 0h; #P<0.05 vs. Veh and †P<0.05 versus respective CARTp + Veh.