

Published in final edited form as:

*Neuron*. 2014 November 19; 84(4): 821–834. doi:10.1016/j.neuron.2014.10.003.

## Descending Control of Itch Transmission by the Serotonergic System via 5-HT<sub>1A</sub>-Facilitated GRP-GRPR Signaling

Zhong-Qiu Zhao<sup>1,2,†</sup>, Xian-Yu Liu<sup>1,2,†</sup>, Joseph Jeffry<sup>1,2</sup>, W.K. Ajith Karunarathne<sup>2,9</sup>, Jin-Lian Li<sup>6</sup>, Admire Munanairi<sup>1,2</sup>, Xuan-Yi Zhou<sup>1,11</sup>, Hui Li<sup>1,2,6</sup>, Yan-Gang Sun<sup>2,12</sup>, Li Wan<sup>1,2,14</sup>, Zhen-Yu Wu<sup>6</sup>, Seungil Kim<sup>2,10</sup>, Fu-Quan Huo<sup>1,2,13</sup>, Ping Mo<sup>1,2,8</sup>, Devin M Barry<sup>1,2</sup>, Chun-Kui Zhang<sup>6</sup>, Ji-Young Kim<sup>2</sup>, N. Gautam<sup>2,3</sup>, Kenneth J. Renner<sup>7</sup>, Yun-Qing Li<sup>6</sup>, and Zhou-Feng Chen<sup>1,2,4,5,\*</sup>

<sup>1</sup>Center for the Study of Itch, Washington University School of Medicine, St. Louis, MO 63110, U.S.A

<sup>2</sup>Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A

<sup>3</sup>Department of Genetics, Washington University School of Medicine, St. Louis, MO 63110, U.S.A

<sup>4</sup>Department of Psychiatry, Washington University School of Medicine, St. Louis, MO 63110, U.S.A

<sup>5</sup>Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A

<sup>6</sup>Department of Anatomy, Histology and Embryology, K. K. Leung Brain Research Centre, The Fourth Military Medical University, Xi'an 710032, P.R. China

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\*Correspondence to: chenz@wustl.edu.

<sup>9</sup>Present address: Department of Chemistry and Biochemistry, The University of Toledo, Toledo, OH 43606, U.S.A

<sup>10</sup>Present address: Department of Cell and Tissue Biology, Craniofacial and Mesenchymal Biology, University of California, San Francisco, CA 94143, U.S.A

<sup>11</sup>Present address: College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, P.R.China

<sup>12</sup>Present address: Institute of Neuroscience, Academy of Chinese Science, Shanghai 200031, P.R. China

<sup>13</sup>Present address: Department of Physiology and Pathophysiology, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi 710061, P. R. China

<sup>14</sup>Present address: Department of Anesthesiology, The Second Affiliated Hospital, Guangzhou Medical University, Guangzhou 510260, P.R. China

<sup>†</sup>These authors contributed to the work equally

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### SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table and Supplemental Experimental Procedures

Figure S1. Reduced Spinal 5-HT Level after 5,7-DHT Injection and Enhanced Central 5-HT Concentrations by Systemic 5-HTP Administration, Related to Figure 1

Figure S2. 5-HT<sub>1A</sub> Antibody Is Specific, Related to Figure 3

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Figure S4. GRP, 8-OH-DPAT and WAY100635 Are Specific, Related to Figure 4

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<sup>7</sup>Department of Biology, University of South Dakota, Vermillion, SD 57069, U.S.A

<sup>8</sup>Department of Anesthesiology, the Affiliated Nanhai Hospital of Southern Medical University, Foshan, 528000, P.R. China

## SUMMARY

Central serotonin (5-HT) modulates somatosensory transduction, but how it achieves sensory modality-specific modulation remains unclear. Here we report that enhancing serotonergic tone via administration of 5-hydroxytryptophan potentiates itch sensation, whereas mice lacking 5-HT or serotonergic neurons in the brainstem exhibit markedly reduced scratching behavior. Through pharmacological and behavioral screening, we identified 5-HT<sub>1A</sub> as a key receptor in facilitating gastrin-releasing peptide (GRP)-dependent scratching behavior. Co-activation of 5-HT<sub>1A</sub> and GRP receptors (GRPR) greatly potentiates subthreshold, GRP-induced Ca<sup>2+</sup> transients and action potential firing of GRPR<sup>+</sup> neurons. Immunostaining, biochemical and biophysical studies suggest that 5-HT<sub>1A</sub> and GRPR may function as receptor heteromeric complexes. Furthermore, 5-HT<sub>1A</sub> blockade significantly attenuates, whereas its activation contributes to, long-lasting itch transmission. Thus, our studies demonstrate that the descending 5-HT system facilitates GRP-GRPR signaling via 5-HT<sub>1A</sub> to augment itch-specific outputs and a disruption of crosstalk between 5-HT<sub>1A</sub> and GRPR may be a useful anti-pruritic strategy.

## Introduction

Somatosensory integration and transduction in the spinal cord and the trigeminal brainstem are subject to monoaminergic modulation. As an integral part of an animal's adaptive response to an ever-changing environment, facilitatory and inhibitory modulation of somatosensory neural circuits is crucial for the maintenance of homeostasis. Monoaminergic modulation of neural circuits serves an important role in regulating behavioral responses to somatosensory inputs. One notable example is that an array of monoamine neuromodulators produced by supraspinal regions is important for the modulation of nociceptive transmission (Basbaum and Fields, 1984; Suzuki et al., 2004). Given the fundamental role of central modulation in sensory transduction, one can presume that itch sensation is similarly modulated at several levels. Imaging studies in human subjects indicated that pain-mediated inhibition of itch is associated with an activity level of the midbrain regions such as periaqueductal gray matter (PAG), an area known for pain modulation (Mochizuki et al., 2003). Experiments in humans also indicated that noxious counterirritants reduce itch sensation (Murray and Weaver, 1975; Ward et al., 1996; Yosipovitch et al., 2007). Interruption of the upper cervical spinal cord of rodents attenuated dry skin-induced itch, suggesting an involvement of the descending spinal pathway in itch responses (Akiyama et al., 2011).

G protein-coupled receptors (GPCRs), or seven trans-membrane proteins, constitute a large repertoire of cellular sensors required for transducing sensory signals from the skin to the brain (Jeffry et al., 2011; Julius and Nathans, 2012). Gastrin-releasing peptide receptor (GRPR) is a G<sub>q</sub> protein-coupled receptor that belongs to the mammalian bombesin receptor family (Jensen et al., 2008; Kroog et al., 1995). GRPR is expressed in laminae I-II of spinal

cord neurons and has an important role in the transmission of pruritogenic information (Liu et al., 2011; O'Donohue et al., 1984; Sun and Chen, 2007; Sun et al., 2009). Gastrin-releasing peptide (GRP), an endogenous neuropeptide for itch, is expressed in a subset of dorsal root ganglion (DRG) and trigeminal ganglion (TG) cells and mediates GRPR activation in the spinal cord (Sun and Chen, 2007; Takanami et al., 2014; Zhao et al., 2013b). While confusion has arisen about GRP expression in DRG neurons, the issue has recently been clarified (Liu et al., 2014; Takanami et al., 2014; Zhao et al., 2013b; Zhao et al., 2014). The GRP-GRPR pathway is primarily engaged in transducing nonhistaminergic acute itch sensation, and may play a relatively minor role in histaminergic itch (Akiyama et al., 2013; Sun et al., 2009; Zhao et al., 2014). Enhanced GRP and GRPR expression is positively correlated with the intensity of chronic itch manifested by increased scratching bouts of animals (Nattkemper et al., 2013; Tominaga et al., 2009; Zhao et al., 2013b). Conversely, loss of GRPR or GRP markedly reduces chronic itch (Lagerstrom et al., 2010; Zhao et al., 2013b), demonstrating the importance of GRPR in the development and maintenance of chronic itch. Despite these studies, molecular mechanisms by which the function of GRPR is modulated remain elusive.

Serotonergic neurons reside principally in the brainstem raphe nuclei and modulate a myriad of behavioral and physiological functions through descending and ascending pathways (Barnes and Sharp, 1999; Dillon et al., 2004). Serotonergic neurons in the rostral ventromedial medulla (RVM), including nucleus raphe magnus (NRM) and surrounding reticular formation, project to both the trigeminal nucleus caudalis (SpVc) and the dorsal horn of the spinal cord and modulate nociceptive transmission in a bidirectional fashion (Basbaum and Fields, 1984; Millan, 2002; Ossipov et al., 2010; Suzuki et al., 2004). In contrast to numerous studies of serotonergic modulation of pain, little is known about the involvement of central 5-HT and receptor mechanisms in itch modulation. In this study, we tested the hypothesis that 5-HT signaling has a pivotal role in itch modulation.

## RESULTS

### Central Serotonin Facilitates Itch Transmission Elicited by Chloroquine

To evaluate the involvement of the central serotonergic system in modulation of itch transmission, we first examined the scratching behavior of *Lmx1b<sup>fl/fl</sup>* mice to intradermal injection (i.d.) of chloroquine (CQ), a representative nonhistaminergic pruritogen that acts, in part, via a Mas-related GPCR (MrgprA3) in sensory neurons and GRPR in the spinal cord (Liu et al., 2009; Sun and Chen, 2007). LMX1B is a LIM homeodomain-containing transcription factor that is required for the development of all central 5-HT neurons (Ding et al., 2003). We previously generated conditional *Lmx1b<sup>fl/fl</sup>* mice in which *Lmx1b* is conditionally ablated in *Pet1*-expressing serotonergic neurons in the raphe nuclei. As a result, all central 5-HT neurons of *Lmx1b<sup>fl/fl</sup>* mice were ablated due to lack of *Lmx1b* (Zhao et al., 2006). However, these mice survive and exhibit normal motor function and thus represent a unique genetic model for investigating the role of central 5-HT neurons in diverse physiological functions (Zhao et al., 2007a; Zhao et al., 2007b). Compared with wild-type (WT) mice, CQ-evoked scratching responses were significantly reduced in *Lmx1b<sup>fl/fl</sup>* mice (Figure 1A), demonstrating that central 5-HT neurons exert a constitutive

tone for positively modulating itch transmission. To determine whether the decrease was specifically due to a loss of 5-HT or whether other factors originating in central 5-HT neurons were involved, we generated mice lacking tryptophan hydroxylase 2 (*Tph2*) that encodes the rate limiting enzyme for 5-HT synthesis in the brain (Walther et al., 2003) using gene targeting strategy (Figure 1B). *Tph2*<sup>-/-</sup> mice showed no expression of *Tph2* and 5-HT in the central nervous system but maintained normal presumptive 5-HT neurons (Figure 1B) (Kim et al., 2014; Liu et al., 2011). *Tph2*<sup>-/-</sup> mice displayed a significant reduction in scratching response after i.d. injection of CQ in nape models (Figure 1C), recapitulating the phenotype of *Lmx1b*<sup>ff/p</sup> mice. Since 5-HT signaling in the brain and the spinal cord may contribute differentially to the modulation of itch sensation, next we asked whether impaired itch sensation observed in *Lmx1b*<sup>ff/p</sup> mice and *Tph2*<sup>-/-</sup> mice may be ascribed to a blockage of the descending 5-HT pathway. We selectively degenerated spinal 5-HT<sup>+</sup> terminals in the spinal cord by an intrathecal injection (i.t.) of 5,7-dihydroxytryptamine (5,7-DHT) following pretreatment with desipramine to prevent the transport of 5,7-DHT into noradrenergic and dopaminergic terminals (Bjorklund et al., 1975). Two weeks after 5,7-DHT injection when spinal 5-HT<sup>+</sup> fibers were depleted (Figure S1A), mice exhibited an attenuated scratching response to CQ (Figure 1D), mimicking the phenotype of *Lmx1b*<sup>ff/p</sup> mice and *Tph2*<sup>-/-</sup> mice. These loss-of-function studies confirm the contribution of descending 5-HT signaling to the modulation of itch sensation.

To complement the loss-of-function approaches, we next determined whether we could rescue the attenuated itch transmission of *Tph2*<sup>-/-</sup> mice and *Lmx1b*<sup>ff/p</sup> mice using an intraperitoneal (i.p.) injection of the 5-HT precursor, 5-hydroxytryptophan (5-HTP), to boost 5-HT tone. Exogenous 5-HTP can be decarboxylated to 5-HT by aromatic L-amino acid decarboxylase (AADC), thereby by-passing the need for TPH2 for 5-HT synthesis (Figure 1B) (Birdsall, 1998). Indeed, immunohistochemical staining (IHC) revealed abundant 5-HT staining in the spinal cord of *Tph2*<sup>-/-</sup> mice after 5-HTP injection (Figure 1E, top panel). In contrast, *Lmx1b*<sup>ff/p</sup> mice treated with 5-HTP failed to produce 5-HT in the brain (Figure 1E, lower row). High performance liquid chromatography (HPLC) analysis also revealed a significant increase in 5-HT concentrations and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the brain and spinal cord of *Tph2*<sup>-/-</sup> and wild-type mice treated with 5-HTP (Figure S1B-S1E). These studies demonstrate a successful conversion of 5-HT from 5-HTP in the absence of TPH2 and that 5-HT synthesis is critically dependent on 5-HT neurons which express AADC. We next examined scratching responses of *Tph2*<sup>-/-</sup> mice after 5-HTP injection and found that 5-HTP injection indeed restored normal scratching responses of *Tph2*<sup>-/-</sup> mice to CQ (Figure 1F). However, the same treatment failed to enhance CQ-elicited scratching in *Lmx1b*<sup>ff/p</sup> mice (Figure 1G). This finding complements the results from the 5,7-DHT lesion study suggesting that 5-HT is important in the facilitation of CQ-induced itch and supports the notion that AADC in 5-HT neurons is required for catalyzing the conversion of 5-HTP into 5-HT. These results also demonstrate that peripheral 5-HT is not involved in modulation of itch transmission. Consistently, WT mice treated with 5-HTP showed a dramatic increase of CQ-elicited scratching (Figure 1H). Since CQ-elicited itch is dependent on GRP-GRPR signaling (Sun and Chen, 2007), we reasoned that 5-HT might synergistically act with GRP to enhance the function of GRPR. We tested this possibility by examining the GRP-induced scratching (GIS) with an intracisternal injection in mice treated

with 5-HTP. Indeed, GIS was markedly potentiated by 5-HTP (Figure 1I), revealing a positive correlation between the activity of GRPR and central 5-HT tone. Importantly, 5-HTP injection did not affect the scratching response-induced by CQ in *Grpr* KO mice (Figure 1J). Taken together, we conclude that spinal 5-HT signaling facilitates itch transmission through GRPR.

### 5-HT1A Mediates 5-HT-Dependent Facilitation of GRPR Function

To identify the 5-HT receptor subtype that mediates the facilitating effect of descending 5-HT on itch transmission, we carried out pharmacological and behavioral screening by injecting a variety of 5-HT receptor agonists and GRP into the spinal cord of WT mice. GRP at 0.01 nmol was used because this dose was insufficient to elicit scratching behavior greater than vehicle (Figure 2A). The use of a minimal concentration of GRP serves to enhance the sensitivity of screening for identifying 5-HT receptor agonists that may potentiate GRP action. Of all the agonists tested for the 5-HT receptor subtypes, only R-(+)-8-OH-DPAT (DPAT), a 5-HT1A agonist, showed a robust facilitating effect on GIS (Figure 2A) and the effect of DPAT is dose-dependent (Figure 2B). Notably, i.t. DPAT alone failed to induce scratch behavior (Figure 2A). Although DPAT is also a partial agonist for 5-HT7 receptor, it is unlikely that 5-HT7 is involved since AS-19, an agonist for 5-HT7, did not enhance GIS significantly (Figure 2A). Furthermore, agonists for other 5-HT receptor subtypes failed to increase GIS (Figure 2A). We next employed WAY100635 (WAY), a highly specific 5-HT1A antagonist, to verify whether the robust scratching response elicited by DPAT/GRP is mediated by 5-HT1A. Pretreatment of mice with WAY (10 µg, i.t.) for 5 min nearly abolished scratching behavior evoked by DPAT/GRP co-injection (Figure 2C). Importantly, DPAT failed to enhance scratching behavior in *Htr1a*<sup>-/-</sup> mice and *Grpr* KO mice (Figure 2D). These data demonstrate that activation of spinal 5-HT1A is required for the facilitation of GRPR function in itch transmission.

Since an elevated 5-HT level facilitates itch elicited by CQ, it is likely that 5-HT1A activation may facilitate CQ-elicited itch as well. Indeed, CQ-induced scratching behavior was greatly enhanced by i.t. DPAT (Figure 2E), mimicking the effect of 5-HTP (Figure 1H). Conversely, pre-injection of WAY markedly attenuated CQ-induced scratching behavior (Figure 2F). Furthermore, CQ-induced scratching behavior was significantly attenuated after spinal siRNA knockdown of *Htr1a* (Figure 2G), which was functionally confirmed by reduced facilitatory effect of DPAT on GIS (Figure 2H). These results suggest that activation of spinal 5-HT1A is important for mediating descending 5-HT signaling to facilitate the function of GRPR in prurceptive transmission.

### Descending 5-HT Terminals Contact GRPR<sup>+</sup> Neurons and co-Expression of 5-HT1A and GRPR in the Spinal Cord

5-HT terminals mainly originated from the NRM are densely distributed in the superficial part (laminae I and II) of the dorsal spinal cord and SpVc of the brainstem (Li et al., 1997). To examine whether 5-HT<sup>+</sup> terminals make connections with GRPR<sup>+</sup> neurons in the dorsal spinal cord, we performed double immunohistochemistry (IHC) for 5-HT and eGFP in GRPR-eGFP mice. Indeed, numerous 5-HT<sup>+</sup> fibers and GRPR<sup>+</sup> neurons were detected in the superficial laminae of the dorsal spinal cord, and 5-HT<sup>+</sup> fibers overlap with all GRPR<sup>+</sup>

neurons (Figure 3A-3F). Synaptic connections between 5-HT<sup>+</sup> terminals and GRPR<sup>+</sup> neurons were further examined by electron microscopy using a double-immunolabeling method (Li et al., 1997) for 5-HT and eGFP. In the lumbar cord, both 5-HT<sup>+</sup> terminals identified by the nanogold–silver enhancement and eGFP (GRPR)<sup>+</sup> neurons revealed by the immunoperoxidase products were observed to distribute in the same pattern as found by our double IHC (Figure 3A-3F). 5-HT<sup>+</sup> terminals form synapses with GRPR<sup>+</sup> dendritic profiles (Figures 3G and 3H). Of the synaptic types characterized (Uchizono, 1965), 85.5% (71/83) were symmetric synapses (Figure 3G), while 14.5% (12/83) were asymmetric ones (Figure 3H). Therefore, serotonergic descending terminals predominantly make symmetrical synapses with GRPR<sup>+</sup> neurons in the dorsal spinal cord.

One prerequisite for 5-HT<sub>1A</sub> and GRPR crosstalk is the co-expression of two partner receptors in the same neurons (Pin et al., 2007). The expression of 5-HT<sub>1A</sub> is heavily concentrated in the dorsal horn of the spinal cord (Zhang et al., 2002). To examine whether 5-HT<sub>1A</sub> is also expressed in GRPR<sup>+</sup> neurons, we performed double IHC of 5-HT<sub>1A</sub> and GFP in the spinal cord of GRPR-eGFP mice using anti-GFP antibody and anti-5-HT<sub>1A</sub> antibody (Figure S2). 5-HT<sub>1A</sub> is broadly expressed in the dorsal horn (Figures 3I-3N). Double IHC revealed that the overwhelming majority (91%) of GFP-expressing cells in the dorsal spinal cord were co-stained with 5-HT<sub>1A</sub> (Figure 3N). Moreover, we examined the co-expression of *Htr1a* and *Grpr* in GRPR-eGFP neurons using single cell RT-PCR (Figure S3B) and found *Htr1a* mRNA signals in 78% (7/9) of eGFP<sup>+</sup> neurons (Table S1). Furthermore, all eGFP<sup>+</sup> neurons expressed *Grpr* mRNA (Table S1), validating the usage of eGFP as a marker for GRPR expression in GRPR-eGFP mice.

### Facilitation of GRP-Induced Calcium Signaling by 5-HT<sub>1A</sub> in Spinal GRPR<sup>+</sup> Neurons

GRPR mediates itch sensation through the PLC $\beta$ /IP<sub>3</sub> pathway and intracellular Ca<sup>2+</sup> release (Liu et al., 2011), whereas 5-HT<sub>1A</sub> signals predominantly through the G<sub>i</sub> protein-coupled cAMP pathway (Hoyer et al., 2002). To investigate the functional cross-communication between 5-HT<sub>1A</sub> and GRPR, we examined intracellular Ca<sup>2+</sup> mobilization in dissociated spinal dorsal horn neurons using calcium imaging. As expected, neither 5-HT or DPAT at 10  $\mu$ M (Figure S4A) nor GRP at 5 nM evoked a Ca<sup>2+</sup> response in spinal dorsal horn neurons (Figure 4A). In contrast, co-application of GRP (5 nM) and 5-HT (10  $\mu$ M) produced Ca<sup>2+</sup> transients in 6% of spinal neurons (30/487) (Figure 4A), which cannot be attributed to additive effect. Similarly, DPAT (10  $\mu$ M) also greatly facilitated GRP-induced Ca<sup>2+</sup> signaling, indicating that 5-HT<sub>1A</sub> mediated the effect of 5-HT (Figure 4A). Importantly, spinal neurons of *Grpr* KO mice did not respond to GRP at high concentrations (up to 20 nM) (Figure S4B), indicating that it is GRPR that mediated the intracellular Ca<sup>2+</sup> mobilization in WT neurons. To further examine the involvement of 5-HT<sub>1A</sub> in the observed facilitating effect of 5-HT and DPAT, WAY was used to block 5-HT<sub>1A</sub> prior to GRP+DPAT/5-HT application. Indeed, WAY completely blocked Ca<sup>2+</sup> responses of GRPR<sup>+</sup> neurons upon GRP+DPAT/5-HT incubation, and the responses recovered after WAY was washed out (Figure 4B). WAY did not display a non-specific effect on intracellular Ca<sup>2+</sup> mobilization evoked by GRP alone without 5-HT<sub>1A</sub> activation (Figure S4C). We previously showed that G<sub>q</sub>/PLC $\beta$ /IP<sub>3</sub>R signaling pathway and intracellular Ca<sup>2+</sup> store are essential for GRP-induced Ca<sup>2+</sup> response in HEK 293 cells (Liu et al., 2011). To

further understand the signaling events downstream of 5-HT<sub>1A</sub>/GRPR upon GRP+DPAT stimulation, we first tested the effect of U73122, a PLC inhibitor, and found that U73122, but not its inactive analog U73343, completely abolished GRP+DPAT-induced Ca<sup>2+</sup> mobilization in GRPR<sup>+</sup> neurons (Figure 4C). GRP+DPAT-responding neurons did not respond to the second incubation of GRP+DPAT after the intracellular Ca<sup>2+</sup> store was depleted by 2,5-di-tert-butyl-hydroquinone (DBHQ), a selective and potent inhibitor of endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) (Moore et al., 1987) (Figure 4D). The effects of U73122 and DBHQ were not due to ligand-induced desensitization of 5-HT<sub>1A</sub>/GRPR signaling pathway because GRPR<sup>+</sup> neurons showed comparable Ca<sup>2+</sup> responses upon two consecutive applications of GRP+DPAT with an interval of 30 min wash (Figure S4D). These results provide the first *in vivo* evidence indicating that co-activation of 5-HT<sub>1A</sub> by 5-HT or DPAT facilitates GRP/GRPR-mediated PLC-dependent intracellular Ca<sup>2+</sup> signaling pathway.

### Heteromeric Interactions Between 5-HT<sub>1A</sub> and GRPR *in vitro* and *in vivo*

GPCR heteromeric interactions have been increasingly implicated in conferring GPCRs with expanded functionality (Bouvier, 2001 ; Milligan, 2013). Receptor crosstalk confers neuronal GPCRs with novel signaling and pharmacological properties enhancing the capacity of neural circuits to regulate a wide array of behavioral outputs (Prinster et al., 2005). The co-expression and synergistic effect of 5-HT<sub>1A</sub> and GRPR co-activation raised the possibility that these two receptors may function as receptor heteromeric complexes. To ascertain whether 5-HT<sub>1A</sub> and GRPR may physically interact through receptor heteromerization, we conducted co-immunoprecipitation (Co-IP) experiments using membrane proteins extracted from HEK 293 cells co-expressing HA-5-HT<sub>1A</sub> and Myc-GRPR. Anti-Myc antibody co-immunoprecipitated a band that corresponds to HA-5-HT<sub>1A</sub> (Figure 5A). In a reverse Co-IP experiment, a specific Myc-GRPR band was also detected in anti-HA precipitates (Figure 5B). In contrast, the specific bands were not observed in the precipitates from mixed membrane proteins that were prepared using cells expressing either HA-5-HT<sub>1A</sub> or Myc-GRPR (Figures 5A and 5B), suggesting the presence of constitutive 5-HT<sub>1A</sub>-GRPR complexes in the membrane when these two receptors are co-expressed in the same cells (Figures 5A and 5B). To verify the specificity of Co-IP between HA-5-HT<sub>1A</sub> and Myc-GRPR, we examined whether GRPR would interact with 5-HT<sub>1B</sub>, a 5-HT<sub>1</sub> receptor subtype that is phylogenetically most closely related to 5-HT<sub>1A</sub> (Hoyer et al., 2002). Myc-GRPR immunoreactivity was not detectable in HA-5-HT<sub>1B</sub> precipitates using HEK 293 cells co-expressing HA-5-HT<sub>1B</sub> and Myc-GRPR (Figure 5C). These results suggest that the formation of 5-HT<sub>1A</sub> and GRPR heteromers is likely to be specific. Next, we examined whether 5-HT<sub>1A</sub> and GRPR interact with each other *in vivo* by Co-IP studies using the spinal cord membrane preparations (Liu et al., 2011). A specific 5-HT<sub>1A</sub> band was co-immunoprecipitated with GRPR using mouse anti-GRPR antibodies (Figure 5D), but was not detectable when an irrelevant mouse IgG was used (Figure 5D). Taken together, these results suggest the presence of receptor heteromeric complexes containing 5-HT<sub>1A</sub> and GRPR both *in vitro* and *in vivo*. Moreover, it further confirms that the two receptors are co-expressed in the same neurons in the spinal cord.

### 5-HT1A and GRPR Are Located in Close Proximity

Although Co-IP data suggest a physical association between 5-HT1A and GRPR, it remained unclear whether the receptors themselves interact or if intermediaries such as scaffolding or anchoring proteins were required for receptor association or whether the two receptors may coexist in microdomains (Prezeau et al., 2010). To examine this possibility, we utilized confocal imaging and single-cell subcellular acceptor photobleaching fluorescence resonance energy transfer (FRET) analysis to assess the physical association between 5-HT1A and GRPR receptors (Karunaratne et al., 2013). A major advantage of our confocal set-up is that the analysis of FRET can be performed in subcellular compartments on a single cell level. This allows us to use a defined region of the same cell that is not photobleached as an internal control. The C-terminus of 5-HT1A and GRPR were tagged with fluorescent protein eGFP as the donor and mCherry as the acceptor, respectively. FRET is calculated by time-lapse imaging of donor GFP (488 excitation, 510 emission) and acceptor mCherry (488 excitation, 630 emission) before and after photobleaching the acceptor (mCherry) in a selected region of the plasma membrane (Figure 5E). A plasma membrane region in the same cell which was not photobleached served as controls (Figure 5E). Fast photobleaching ( $0.89 \text{ ms}/\mu\text{m}^2$ ) and slow mobility of several transmembrane receptors on the plasma membrane ensured that during FRET analysis both the donor and acceptor fluorescent proteins remained stationary. After acceptor (5-HT1A-mCherry) photobleaching, donor (GRPR-eGFP) fluorescence intensity was increased due to the loss of energy transfer, suggesting the presence of FRET (Figure 5F and 5G). The FRET loss associated with acceptor photobleaching and subsequent increase in donor fluorescence intensity was  $44.8 \pm 11.2 \%$  (Figure 5F and 5G). In contrast, no detectable FRET signal was observed between 5-HT1B-mCherry and GRPR-eGFP (Figure S6B). Lack of FRET between 5-HT1B and GRPR is consistent with Co-IP results, further supporting the notion that the 5-HT1A-GRPR association is specific. Taken together, these results suggest that 5-HT1A and GRPR are in close proximity on the plasma membrane, which may facilitate crosstalk between the two receptors.

### Activation of 5-HT1A Potentiates the Excitability of GRPR<sup>+</sup> Neurons

We next evaluated the effect of 5-HT1A activation on GRP-induced excitation of GRPR<sup>+</sup> neurons by whole-cell patch-clamp recordings of on GRPR-eGFP neurons using spinal cord slice preparations, hereafter referred to as GRPR<sup>+</sup> neurons (Zhao et al., 2013a). We first measured changes in membrane potential and input resistance of GRPR<sup>+</sup> neurons in response to GRP, DPAT and 5-HT. A bath application of GRP induced a subthreshold membrane depolarization (Figures 6A and 6E). In contrast, DPAT and 5-HT hyperpolarized GRPR<sup>+</sup> neurons as expected (Figures 6B, 6C and 6E). Thus, activation of GRPR and 5-HT1A alone appeared to have opposing effects on the excitability of GRPR<sup>+</sup> neurons in the spinal cord. To determine whether DPAT or 5-HT could facilitate GRP-dependent excitation, we examined the effect of a co-application of GRP and DPAT or 5-HT on GRPR<sup>+</sup> neurons. Importantly, a co-application of GRP+DPAT or GRP+5-HT not only masked the hyperpolarization observed in response to DPAT or 5-HT alone, but also induced a larger magnitude of depolarization that often resulted in action potential (AP) firing (Figure 6D). GRP and GRP + DPAT treatments significantly increased input resistance compared to control, whereas DPAT alone significantly decreased input



resistance compared to control (Figures 6F and 6G). These data revealed that GRP-induced subthreshold membrane depolarization not only counteracted the hyperpolarizing effect of DPAT but was also potentiated by DPAT with a net increase in the excitability of GRPR<sup>+</sup> neurons.

### A Blockade of 5-HT<sub>1A</sub> Attenuates Chronic Itch

In light of the facilitatory effect of 5-HT<sub>1A</sub> on GRPR signaling and CQ-elicited itch, next we asked whether 5-HT<sub>1A</sub> could modulate long-lasting scratching behavior using two distinct chronic itch models that are dependent on enhanced GRP/GRPR signaling for maintaining long-lasting itch transmission (Zhao et al., 2013b). First, we tested the effect of 5-HT<sub>1A</sub> activation on spontaneous scratching behavior of BRAF<sup>Nav1.8</sup> mice, in which the BRAF kinase in sensory neurons expressing the sodium channel Nav1.8 was selectively activated by genetically replacing the WT *Braf* gene with a kinase activated one (V600E) (Zhao et al., 2013b). BRAF<sup>Nav1.8</sup> mice progressively developed spontaneous scratching behavior accompanied by skin lesions as a result from increased expression of a cohort of itch-related genes, including GRP in sensory neurons and GRPR in the spinal cord (Zhao et al., 2013b). Although WT mice failed to exhibit scratching behavior after i.t. DPAT alone, the spontaneous scratching behavior of BRAF<sup>Nav1.8</sup> mice was significantly enhanced (Figure 7A). Likewise, DPAT also significantly enhanced the chronic scratching behavior induced by dry-skin (xerosis) (Figure 7B), a skin condition often associated with pruritus (Miyamoto et al., 2002). The enhanced scratching responses in mice with chronic itch by DPAT suggests that 5-HT<sub>1A</sub> is likely to be constitutively primed for tonic activation by enhanced release of 5-HT. To test this, we measured the levels of 5-HT and 5-HIAA in mice with chronic itch using HPLC. Moreover, the levels of 5-HT were significantly elevated in the hindbrain of BRAF<sup>Nav1.8</sup> mice and cervical spinal cord of dry-skin mice (Figure S7A). The levels of 5-HIAA were also significantly elevated in hindbrain and cervical spinal cord of BRAF<sup>Nav1.8</sup> mice, suggesting enhanced activity of 5-HT in these regions (Figure S7B). Then, we examined whether WAY may attenuate chronic itch. Similar to its inhibitory effect on CQ-elicited itch, i.t. WAY significantly attenuated spontaneous scratching behaviors of BRAF<sup>Nav1.8</sup> mice (Figure 7C) and dry skin mice (Figure 7D). Importantly, injection of saline had no significant effect on spontaneous scratching behaviors of BRAF<sup>Nav1.8</sup> mice or dry skin mice (Figures 7C and 7D). Furthermore, *Tph2*<sup>-/-</sup> mice also displayed deficits in spontaneous scratching behaviors under dry-skin condition (Figure 7E) and WT mice showed attenuated scratching behavior after knock-down of spinal 5-HT<sub>1A</sub> by i.t. *Htr1a* siRNA (Figure 7F). Thus, these data support the notion that 5-HT<sub>1A</sub> is constitutively activated in the setting of chronic itch and its activation by descending 5-HT is required for maintaining long-lasting itch transmission.

## DISCUSSION

### The Role of 5-HT<sub>1A</sub> in the Modulation of Itch Transmission

Using both loss- and gain-of-function, genetic, pharmacological, behavioral and electrophysiological examinations, we demonstrate that central 5-HT signaling is essential for facilitating itch transmission and this function is mediated by the 5-HT<sub>1A</sub> receptor. Decreased itch transmission in mice lacking 5-HT is unlikely to be due to developmental

deficits because we were able to restore normal itch transmission in *Tph2*<sup>-/-</sup> mice by an administration of exogenous 5-HTP. Furthermore, we demonstrate that central 5-HT neurons are essential for converting 5-HTP into 5-HT since mice lacking these neurons failed to be rescued by exogenous 5-HTP. Our study reveals a previously unrecognized function of 5-HT in sensory modality-specific modulation and demonstrate that 5-HT1A is a principal receptor for mediating 5-HT-dependent modulation of itch transmission. Importantly, the facilitatory role of 5-HT1A is required not only for acute itch but also for long-lasting itch transmission. Therefore, 5-HT1A has a broad role in the modulation of itch transmission.

### 5-HT1A-GRPR Crosstalk Amplifies Itch Signaling

A key finding of our work is that 5-HT1A facilitates itch modulation through its crosstalk with GRPR. Several pieces of evidence such as co-expression, co-IP and FRET data imply a physical association between 5-HT1A and GRPR in the same cells. While further studies are needed to explain whether the existence of 5-HT1A-GRPR heteromeric complexes is a prerequisite for the initiation of signaling crosstalk, it is tempting to speculate that the spatial proximity of 5-HT1A and GRPR may permit specific heteromeric crosstalk without accidental engagement of unrelated signaling pathways in GRPR<sup>+</sup> neurons. A close proximity with 5-HT1A may enable GRPR to process information via pairing with specific GPCRs in a real time manner. 5-HT1A-dependent facilitation requires co-activation of GRPR by exogenous GRP. Although co-application of GRP is not required in chronic itch models for 5-HT1A-dependent facilitation, it is presumed that there is a constitutive release of GRP from primary afferents to prime GRPR for 5-HT modulation because GRP expression is significantly up-regulated in chronic itch conditions and GRP blocker attenuated spontaneous scratching behaviors (Liu et al., 2014; Nattkemper et al., 2013; Zhao et al., 2013b).

The mode of 5-HT1A or GRPR activation in a single receptor paradigm clearly differs from that of the two receptor paradigm. The fact that DPAT by itself is unable to elicit scratching behavior implies that 5-HT1A cannot transmit itch information directly. In contrast, GRP at a low dose that is insufficient to elicit either a calcium response (5 nM) or scratching behavior (0.01 nmol) can do so only if 5-HT1A is simultaneously activated. Since DPAT alone fails to produce calcium spikes, it is unlikely that DPAT alone is capable of exciting GRPR<sup>+</sup> neurons. On the contrary, DPAT causes hyperpolarization of GRPR<sup>+</sup> neurons. Postsynaptic 5-HT1A activation predominantly hyperpolarizes dorsal horn neurons to dampen neuronal excitability and synaptic transmission (Grudt et al., 1995; Yoshimura and Furue, 2006). Remarkably, the hyperpolarizing response of GRPR<sup>+</sup> neurons produced by DPAT was completely masked by GRP. When GRP and DPAT were co-applied, together they were able to induce action potential firing. This response was not observed by an application of either GRP or DPAT alone. Thus, interactions between 5-HT1A and GRPR alter the pharmacological and physiological properties of either of the receptors and confer a novel functionality to 5-HT1A (Figure 8A). While activation of G<sub>i</sub>-coupled 5-HT1A is usually inhibitory via the cAMP pathway, the receptor may facilitate the responsiveness of G<sub>q</sub>-coupled receptors through signaling coupling. Our finding thus uncovers a novel paradigm for exploring facilitatory crosstalk mechanisms between G<sub>i</sub>- and G<sub>q</sub>-coupled

receptor signaling transduction in a highly physiologically relevant context. It is important to note that the present study is only the first step towards understanding of central modulation of itch transmission. Since both heteromeric interactions and/or convergence of intracellular signaling pathways may occur (Prezeau et al., 2010), our studies raise several key questions concerning the underlying mechanisms: What are the interfacing regions for 5-HT<sub>1A</sub>-GRPR heteromeric interaction? Does the cross-talk between 5-HT<sub>1A</sub> and GRPR occur at the receptor or intracellular second messenger levels or both? If intracellular crosstalk is required, does 5-HT<sub>1A</sub> potentiate GRPR signaling by releasing G<sub>βγ</sub> subunits to stimulate G<sub>q</sub>-coupled PLC signaling? What is the respective role of Gi coupled signaling in activation vs. facilitation of GRPR? It will be of great interest to determine where the two signaling pathways might converge to amplify G<sub>q</sub>-dependent itch signaling through either a “switch” or “turn off” of the signaling characteristic of Gi coupled receptors.

### **Opposing Modulation of Itch and Pain by 5-HT Receptor Mechanisms**

Pain and itch have been known as two opposing sensations (Ikoma et al., 2006; Lamotte et al., 2013; Ma, 2010) and, conceivably, may be subject to opposing descending modulation in the spinal cord. Despite some conflicting reports and the pain modality-dependent role of 5-HT<sub>1A</sub> (Bardin, 2011), most studies suggest an inhibitory function for postsynaptic 5-HT<sub>1A</sub> in spinal nociceptive processing, especially in thermal and inflammatory pain (Bardin, 2011; Millan, 2002; Yoshimura and Furue, 2006; Zhao et al., 2007a). The majority of substantia gelatinosa (lamina II) neurons of the dorsal horn and SpVc responded to 5-HT or 5-HT<sub>1A</sub> agonists with a hyperpolarizing membrane current, thereby inhibiting nociceptive processing (Abe et al., 2009; Grudt et al., 1995; Lu and Perl, 2007; Yoshimura and Furue, 2006). In contrast, 5-HT<sub>1A</sub> facilitates itch by enhancing the excitability of GRPR<sup>+</sup> neurons and by inhibiting its canonical pathway. Taken together, 5-HT<sub>1A</sub> has opposing roles in mediating descending 5-HT modulation of itch and pain, and this dual role is exerted through distinct subsets of dorsal horn neurons which express 5-HT<sub>1A</sub> with or without GRPR (Figure 8C). Along with previous studies, our data, therefore, provide the first evidence indicating that itch and pain are subject to opposing modulation of descending 5-HT signaling in the spinal cord.

### **Contribution of Central 5-HT Signaling to Development of the Itch-Scratch Cycle**

What is the probable physiological significance of 5-HT<sub>1A</sub>-mediated central modulation of itch and pain? A cardinal feature of chronic itch associated with a wide spectrum of skin diseases is a vicious itch-scratch cycle (ISC) (Paus et al., 2006; Yosipovitch and Papoiu, 2008)(Figure 8B). Scratching behavior, as a noxious mechanical stimulus, can inhibit itch sensation and spinal projection neurons (Davidson et al., 2009; Yosipovitch et al., 2007). However, it also intensifies skin inflammation which, in turn, provokes more intense itch sensation as well as an uncontrollable urge to scratch. Given the role of postsynaptic 5-HT<sub>1A</sub> in the inhibition of inflammatory pain and spinal nociceptive processing, we suggest a hypothetical model that explains the dual consequences of scratching behavior (Figure 8C). Past studies indicated that activation of the nociceptive pathway in response to somatic and/or noxious stimuli can promote 5-HT release in the spinal cord (Sorkin and McAdoo, 1993; Yaksh and Tyce, 1981). Likewise, vigorous scratching-induced pain can conceivably evoke 5-HT release to inhibit nociceptive processing through 5-HT<sub>1A</sub>-dependent negative

feedback mechanisms (Figure 8B). Concurrently, tonic 5-HT release paradoxically activates 5-HT<sub>1A</sub> in GRPR<sup>+</sup> neurons to potentiate itch transmission (Figure 8C). Thus, the positive and negative feedback controls of pain and itch outputs are likely to contribute to the vicious development of the ISC.

Because cross-signaling of 5-HT<sub>1A</sub>-GRPR heteromers is itch-specific (only when GRPR is activated), a disruption of an interface between 5-HT<sub>1A</sub> and GRPR may alleviate the urge to scratch in chronic itch conditions. The advantage of such a strategy is to permit the normal functioning of 5-HT<sub>1A</sub> and GRPR in other areas of the brain where the receptors are not co-expressed. Unraveling key interfaces required for 5-HT<sub>1A</sub>-GRPR cross-signaling could be a crucial next step. Since GPCR crosstalk may occur at multiple signaling levels, the converging points of G<sub>i</sub>-G<sub>q</sub> cross-signaling may also be substrates for a therapeutic blockade. As enhanced central 5-HT signaling is likely to exacerbate the ISC, an exploration of the disruption of modulatory function of 5-HT<sub>1A</sub> opens an additional avenue for designing novel therapeutics that ameliorate chronic pruritus.

## EXPERIMENTAL PROCEDURES

### Animal Behavior

Adult male C57BL/6J mice, *Lmx1b<sup>fl/fl</sup>* mice (Zhao et al., 2006), *Tph2<sup>-/-</sup>* mice (Kim et al., 2014), *Htr1a<sup>-/-</sup>* mice (Heisler et al., 1998), *Grpr* KO mice (Hampton et al., 1998) and BRAF<sup>Nav1.8</sup> mice (Zhao et al., 2013b) were used for the study. Scratching behaviors were performed as previously described (Sun and Chen, 2007; Zhao et al., 2013b). All animal experiments were performed in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain and were approved by the Animal Studies Committee at Washington University School of Medicine.

### Immunohistochemistry and In Situ Hybridization

IHC and ISH were performed as described (Zhao et al., 2006). For the IHC study, sections were incubated with primary antibodies overnight at 4°C followed by the use of FITC or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch). The following primary antibodies were used: rabbit anti-5-HT (1:5,000, Immunostar), rabbit anti-5-HT-1A (1:200, Santa Cruz) and chicken anti-GFP (polyclonal, 1:500, Aves Labs). For the ISH study, a digoxigenin-labeled cRNA probe was used as described earlier (Zhao et al., 2006). Images were taken using a Nikon Eclipse Ti-U microscope.

### Small Interfering RNA Treatment

*Htr1a* siRNA (Sigma) were delivered to the lumbar region of the spinal cord, twice daily for 6 consecutive days as described previously (Liu et al., 2011). Behavioral testing was carried out 24 h after the last injection.

### Immune-Electron Microscopy

Immune-electron microscopic studies were performed as described (Li et al., 1997; Pang et al., 2006). Briefly, cross sections of lumbar spinal cord of adult GRPR-eGFP mice were double immune-labeled by rabbit anti-5-HT antibody (1:2,000; Incstar Corporation,

Stillwater, MN) and guinea pig anti-GFP antibody (1.5 µg/ml) using immunogold–silver and immunoperoxidase methods, respectively. Furthermore, 50-nm-thick ultrathin sections were examined with a JEM-1400 electron microscope (JEM, Tokyo, Japan). The digital micrographs were captured by VELETA (Olympus, Tokyo, Japan).

## HPLC

The concentrations of monoamines were measured as previously described (Zhao et al., 2006). The resulting values were corrected for volume and expressed as pg of amine per mg of wet tissue or per 100 µL of plasma. For analyzing the effect of 5-HTP injections on indoleamine concentrations, samples were collected one hour after 5-HTP or saline administration.

## Cell Culture and Transfections

Constructs were transfected into HEK 293 cell lines for selection as described (Liu et al., 2011). For FRET experiments, pcDNA3.1/GRPR-eGFP (1 µg/cm<sup>2</sup>) and pcDNA3.1/5-HT1A-mCherry (0.2 µg/cm<sup>2</sup>) were transiently transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen). FRET was done 24 h after transfection.

## Calcium Imaging, Co-IP and Western Blot Analysis

Calcium imaging experiments were performed as described previously (Liu et al., 2011). Experiments were repeated three times and a minimum of 50 cells were included for analysis. Co-IP and Western Blot Analysis were performed as described (Liu et al., 2011). Briefly, solubilized membrane proteins (200 µg) were precipitated with rabbit anti-HA (BD bioscience), mouse anti-Myc (Covance) or mouse anti-GRPR (Abmart) antibodies and TrueBlot™ anti-rabbit or anti-mouse IgG bead slurry (eBioscience). After elution proteins were analyzed using western blot with mouse anti-Myc (1:1,000), rabbit anti-HA (1:1,000), mouse anti-GRPR (1:5,000) or rabbit anti-5-HT1A (1:5,000, Abcam).

## Confocal Subcellular FRET Imaging

The FRET imaging and calculations were performed as described previously (Karunaratne et al., 2013). Basal FRET between eGFP (donor) and mCherry (acceptor) was measured by rapid photobleaching of the acceptor in a defined region of a single cell, whereas the unbleached region was used as the control. Before and after photobleaching, a series of time lapse images were captured with donor excitation-donor emission (DD) and donor excitation-acceptor emission (DA).

## Electrophysiological Recording

Patch-clamp studies were performed as described previously (Jeffry et al., 2009). Slices of the lumbar spinal cord of GRPR-eGFP mice 3–4 weeks of age were prepared for patch clamp recording. Firing patterns were examined by injection of steps of positive current for 500 ms. Input resistance was tested every 20 s for drug induced changes by an injection of negative current (–20 pA). Series resistance was monitored in voltage clamp mode by measuring the instantaneous current in response to small voltage steps. Data were analyzed offline (ClampFit 10) and plotted in Origin 8 graphing software.

## Statistical Analysis

Statistical analyses were performed using Prism 5 (version 5.03, GraphPad Software).  $p < 0.05$  was considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We are grateful to Y.Z Tian, AA. Kim, R. Kim, J. Yin, X. L Meng, Q. Meng, N. Han and Q. F. Li for technical help and members of the Chen laboratory for comments. The project was supported by a NIAMS grant AR056318-01 (Z.F.C), a NIH grant R01 DA019921 (K.J.R.), the National Natural Science Foundation of China grants No. 81371239 and 31371211 (H.L and Y.L) and NIH grants GM069027 and GM080558 (N.G.).

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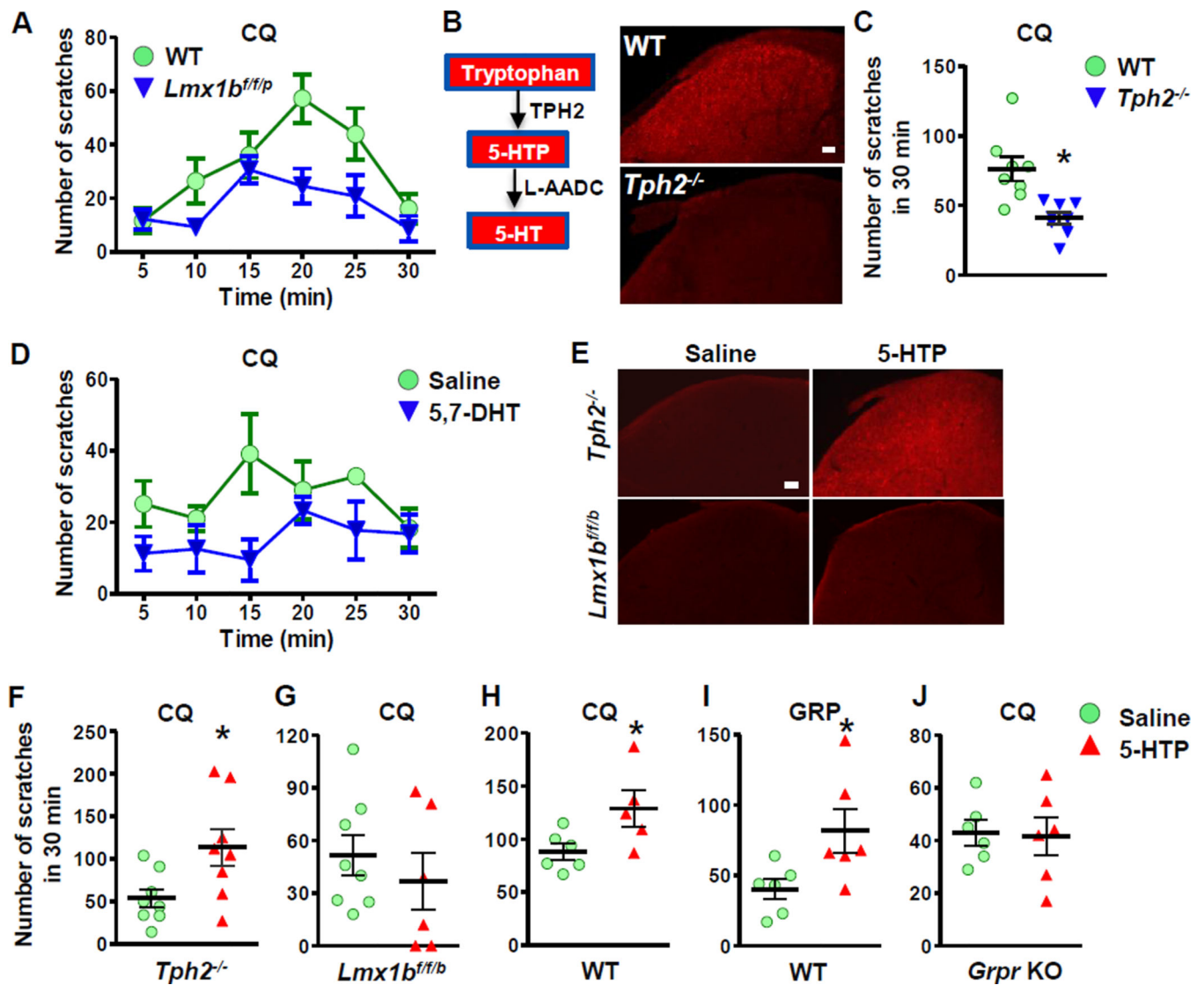
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### Highlights

1. Central 5-HT signaling facilitates itch transmission
2. 5-HT1A potentiates GRPR-mediated itch signaling
3. 5-HT1A and GRPR are present in close proximity
4. Blockade of 5-HT1A function reduces chronic itch



**Figure 1. Central Serotonin Facilitates Itch Transmission**

(A) *Lmx1b<sup>fl/fl</sup>* mice showed deficits in CQ-induced scratching behavior ( $p < 0.05$ ).

(B) Diagram showing synthesis of 5-HT in the brain. IHC images show that spinal 5-HT was not detectable in *Tph2<sup>-/-</sup>* mice.

(C) *Tph2<sup>-/-</sup>* mice exhibited attenuated CQ-induced scratching responses.

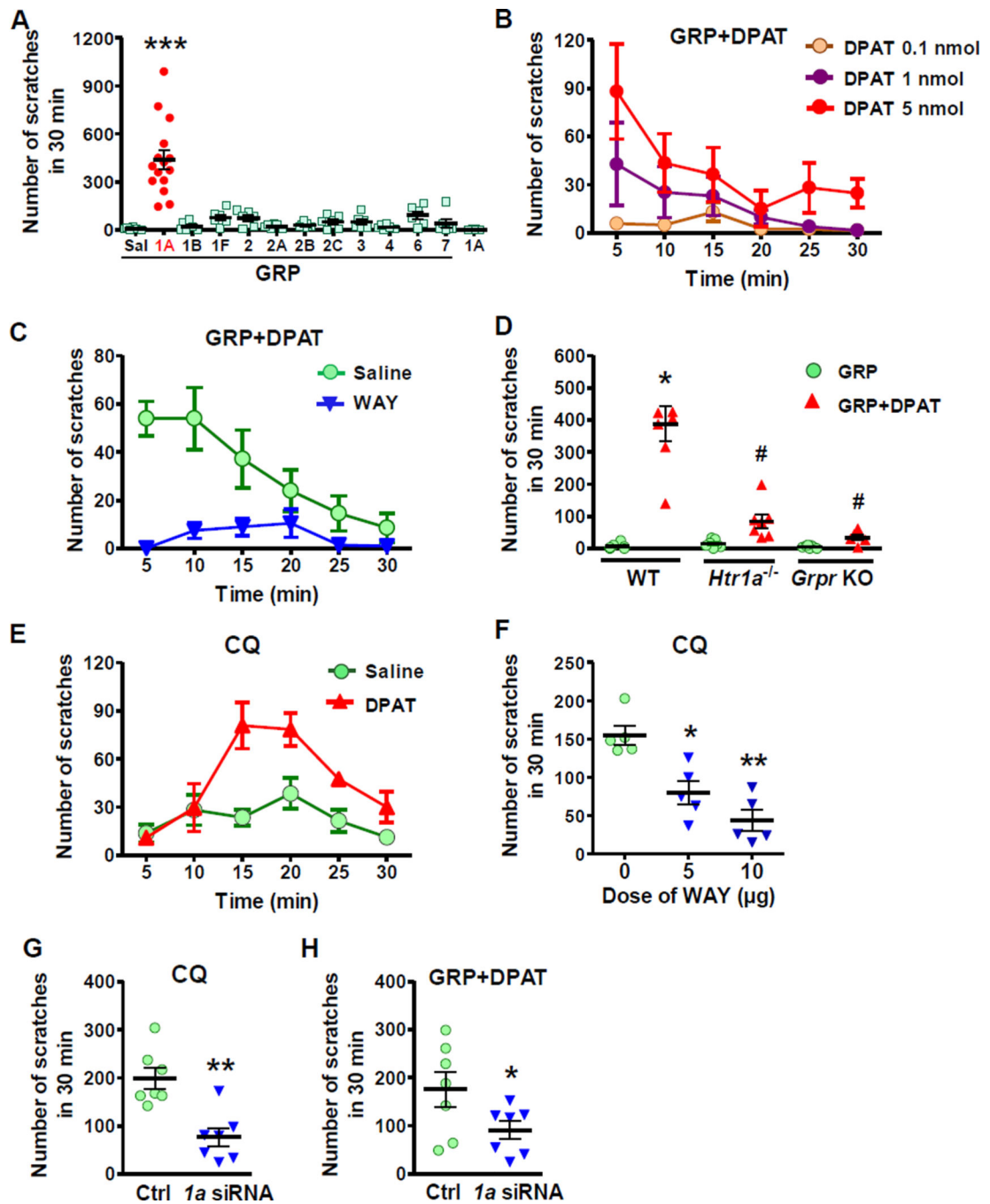
(D) CQ-induced scratching responses were attenuated after 5,7-DHT injection in C57Bl/6J mice ( $p < 0.05$ ).

(E) Injection of 5-HTP (10 mg/kg, i.p.) for 60 min restored dorsal spinal 5-HT in *Tph2<sup>-/-</sup>* mice, but not in *Lmx1b<sup>fl/fl</sup>* mice.

(F and G) Injection of 5-HTP for 30 min rescued CQ-induced scratching behavior in *Tph2<sup>-/-</sup>* mice (F) but not in *Lmx1b<sup>fl/fl</sup>* mice (G).

(H-J) Injection of 5-HTP facilitated scratching behaviors induced by CQ (H) and GRP (I) in C57Bl/6J mice, while *Grpr* KO mice did not respond to 5-HTP injection (J).

Error bars represent SEM. \* $p < 0.05$ , versus WT (C) or saline (F-J), by un-paired  $t$  test in C, F-J or Two-way repeated measures ANOVA in A, D.  $n = 6-9$ . Scale bars: 100  $\mu\text{m}$ . See also Figure S1.



**Figure 2. Serotonin Facilitates Scratching Behavior Through 5-HT1A**

(A) Scratching behaviors evoked by co-injection of GRP and various 5-HT receptor agonists (5 nmol) in WT mice. Sal: saline, 1A: DPAT, 1B: CP 93129, 1F: LY344864, 2:  $\alpha$ -ME-5-HT, 2A: DOI, 2B: BW 723C86, 2C: m-cpp, 3: m-CPBG, 4: RS 67506, 6: EMD 386088, 7: AS-19.

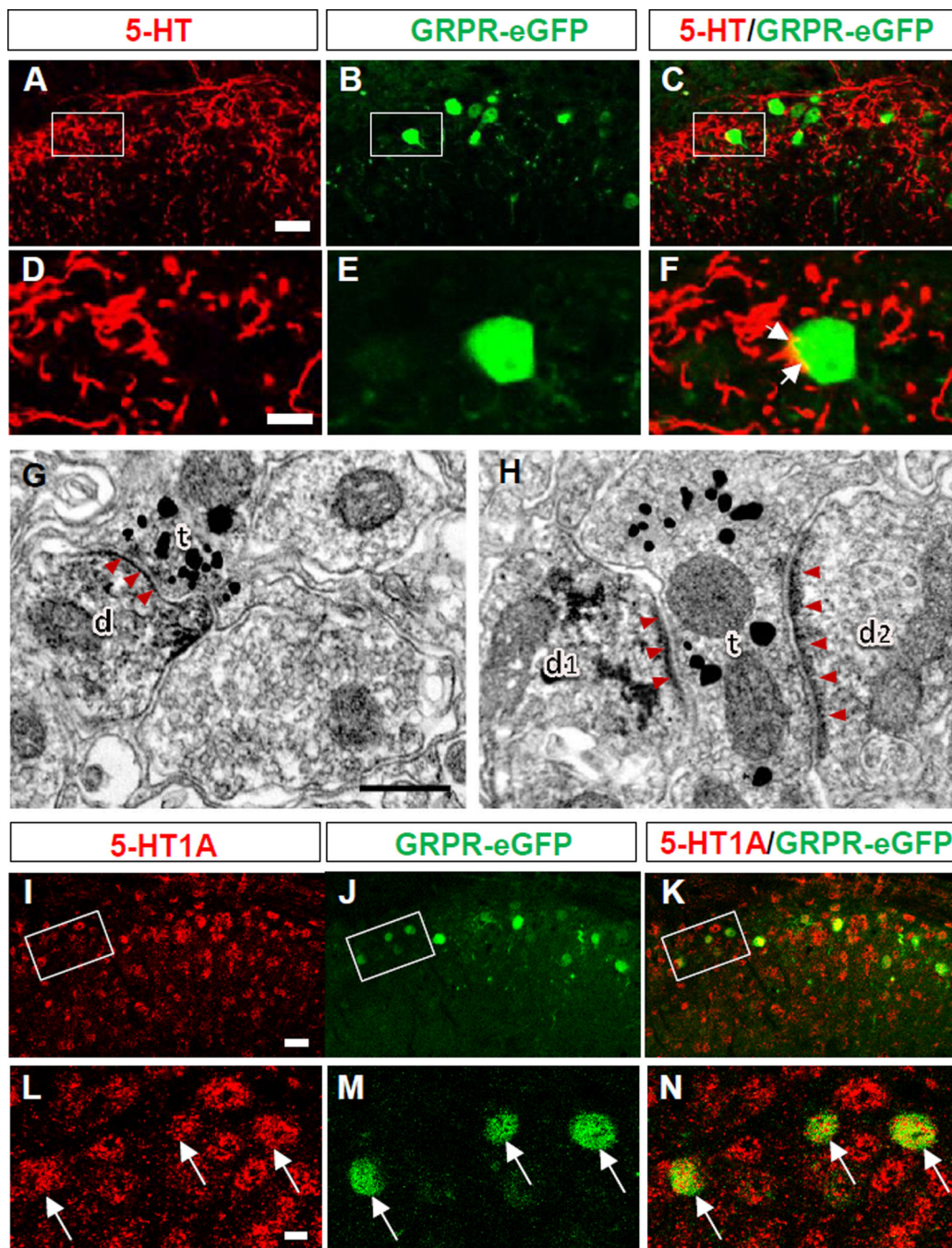
(B) Co-injection of DPAT dose-dependently facilitated GIS ( $p < 0.01$ ).

(C) WAY blocked the facilitatory effect of DPAT on GIS ( $p < 0.01$ ).

(D) Co-injection of DPAT and GRP failed to elicit scratching behavior in *Htr1a*<sup>-/-</sup> mice or *Grpr* KO mice. (E-G) CQ-induced scratching behavior was significantly enhanced by DPAT ( $p < 0.01$ ) (E), while attenuated by WAY (F) and *Htr1a* siRNA (*1a* siRNA) (G).

(H) Facilitatory effect of DPAT on GIS was significantly reduced by *Htr1a* siRNA.

Error bars represent SEM.  $n = 5-9$ . \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus saline (A, D and F) or ctrl (G and H). # $p < 0.05$ , versus DPAT in WT. One-way ANOVA followed by Dunnett's Multiple Comparison Test in A, D and F, two-way repeated measures ANOVA in B, C and E, un-paired *t* test in G and H.



**Figure 3. Co-Expression of GRPR and 5-HT or 5-HT1A in the Spinal Cord**

(A-C) Double immunostaining of 5-HT<sup>+</sup> terminals (red) with GFP (green) in the superficial dorsal horn.

(D-F) High power image of boxed area in A-C. Arrows indicate close contacts between 5-HT<sup>+</sup> terminals and GRPR<sup>+</sup> neuronal cell bodies.

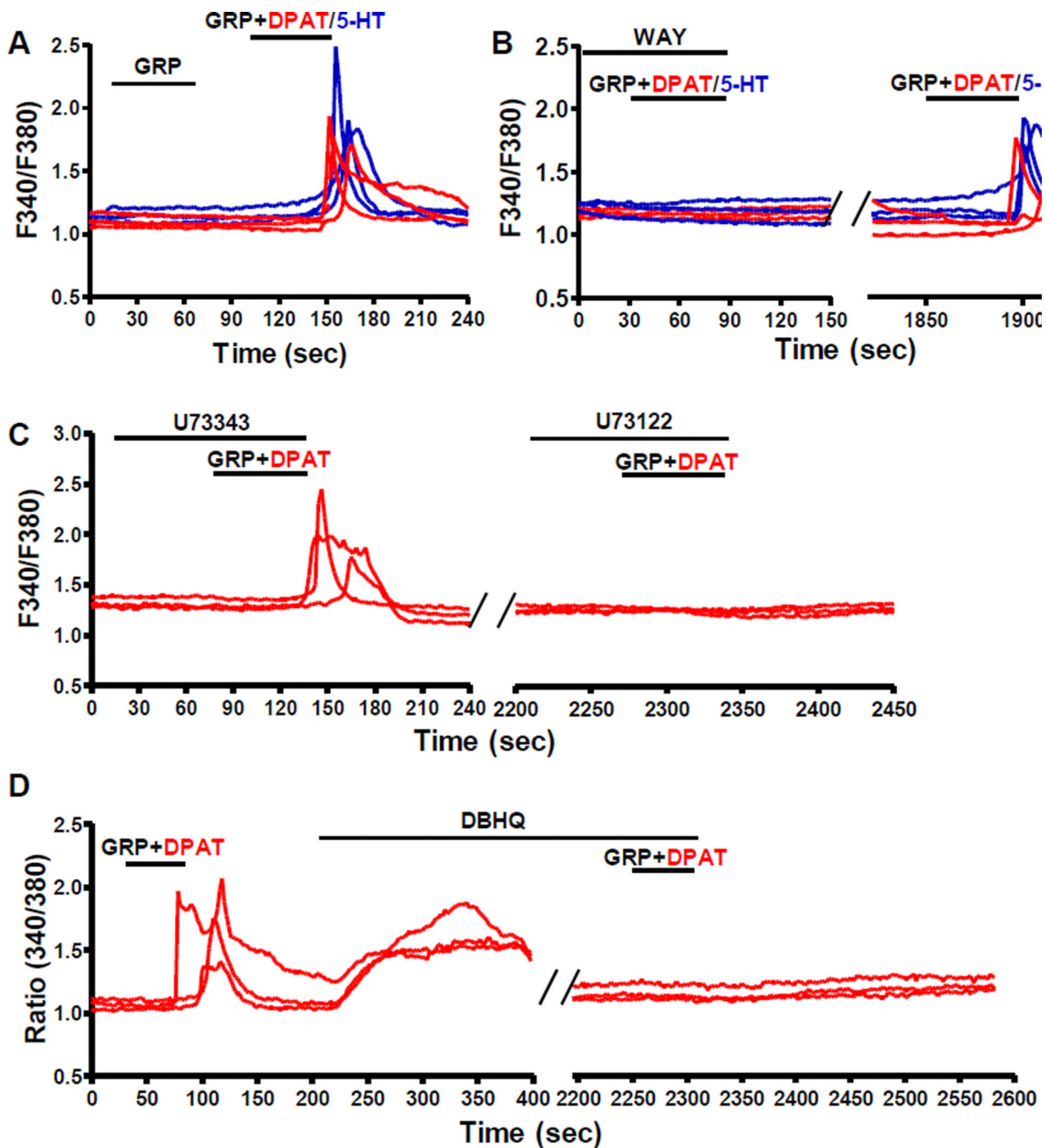
(G, H) 5-HT<sup>+</sup> axon terminals (t; silver grains) make symmetric (G) or asymmetric (H) synaptic contacts with GRPR<sup>+</sup> dendritic profiles (d, d1, d2; DAB reaction products), respectively. Arrow heads indicate post-synaptic membranes.

(I-K) Double immunostaining of 5-HT1A (red) and GFP (green) in the superficial dorsal horn.

(L-N) High power image of boxed area in I-K. Arrows indicate double-stained cells.

Scale bars: 10  $\mu\text{m}$  in A and I, 2.5  $\mu\text{m}$  in D and L, 0.3  $\mu\text{m}$  in G and H. See also Figures S2 and S3.

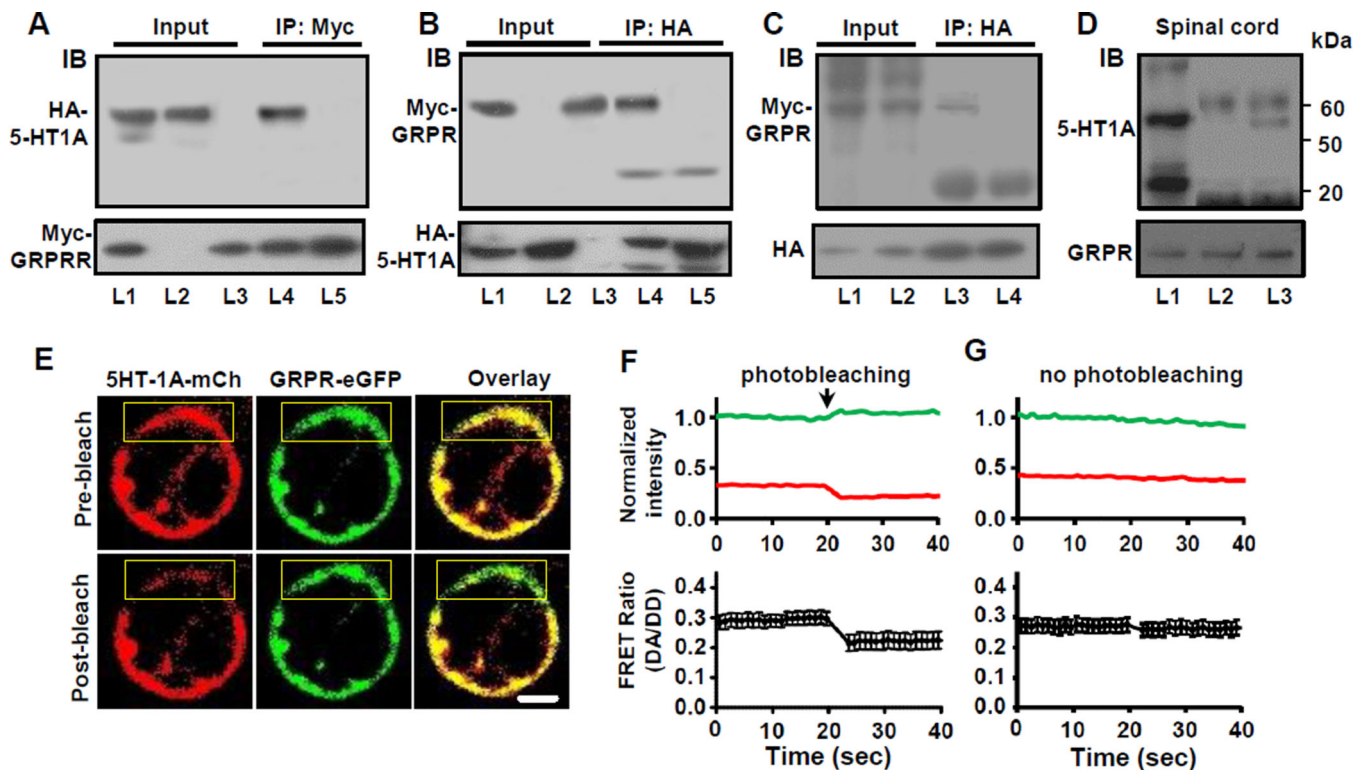




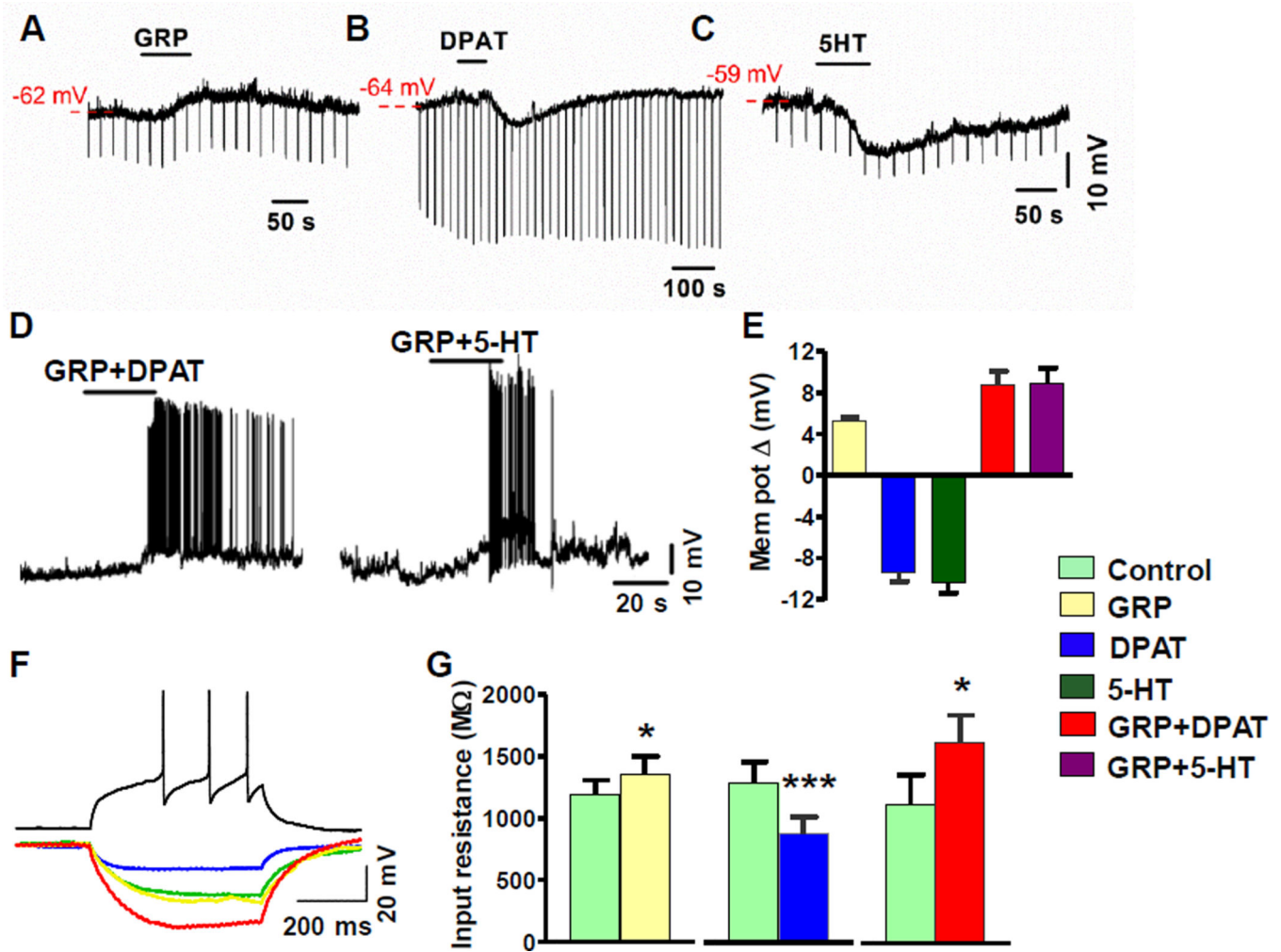
**Figure 4. Co-Activation of 5-HT<sub>1A</sub> Facilitates GRPR Ca<sup>2+</sup> Signaling in Spinal Neurons**  
 (A) Representative traces showing that 5 nM GRP failed to evoke Ca<sup>2+</sup> responses in dissociated WT spinal neurons, while co-application of 5-HT (blue) or DPAT (red) together with GRP (5 nM) induced intracellular Ca<sup>2+</sup> mobilization.  
 (B) WAY completely blocked Ca<sup>2+</sup> responses of spinal neurons evoked by GRP+5-HT (blue) or GRP+DPAT (red), which recovered after WAY was washed out.  
 (C) U73122, but not U73343 blocked intracellular Ca<sup>2+</sup> mobilization induced by GRP +DPAT.  
 (D) DBHQ blocked intracellular Ca<sup>2+</sup> mobilization induced by GRP +DPAT.

(D) GRP+DPAT-evoked intracellular  $\text{Ca}^{2+}$  mobilization vanished after depletion of intracellular  $\text{Ca}^{2+}$  store by DBHQ.

The experiments were repeated for 3 times and at least 200 neurons were analyzed for each experiment. See also Figure S4.



**Figure 5. GRPR and 5-HT1A Form Heteromeric Complexes and Are within Close Proximity** (A and B) Co-IP of HA-5-HT1A and Myc-GRPR. L1 and L4, HA-5-HT1A/Myc-GRPR cells; L2, HA-5-HT1A cells; L3, Myc-GRPR cells; L5, mixture of L2 and L3. (C) Myc-GRPR was not co-immunoprecipitated with HA-5-HT1B. L1, L3, HA-5-HT1A/Myc-GRPR cells; L2, L4, HA-5-HT1B/Myc-GRPR cells. (D) Co-IP of 5-HT1A and GRPR from the spinal cord membrane preparations. L1, input; L2, irrelevant mouse IgG; L3, mouse anti-GRPR. (E) Representative confocal images show one HEK 293 cell co-expressing 5HT1A-mCherry (red) and GRPR-eGFP (green), before and after acceptor (mCherry) photobleaching in selected region of the cell (yellow box). Scale bar, 10  $\mu$ m. (F) Top: Plots showing background subtracted normalized fluorescence intensities of donor emission (green trace, 488 nm excitation, 515 nm emission: DD) and acceptor emission (red trace, 488 nm excitation, 630 nm emission: DA) from selected plasma membrane region in E. Bottom: Averaged FRET ratio (DA/DD) of the photobleached regions in E. (G) Background subtracted normalized fluorescence intensities (top) and averaged FRET ratio (bottom) of non-photobleached plasma membrane regions in E. Number of cells, n = 9–11. Error bars represent SEM. See also Figures S5 and S6.



**Figure 6. Co-Activation of 5-HT1A and GRPR Increased the Excitability of GRPR<sup>+</sup> Neurons**

(A-C) Representative traces show membrane depolarization by GRP (A) and hyperpolarizing response induced by DPAT (B) and 5-HT (C) in current-clamped GRPR<sup>+</sup> neurons.

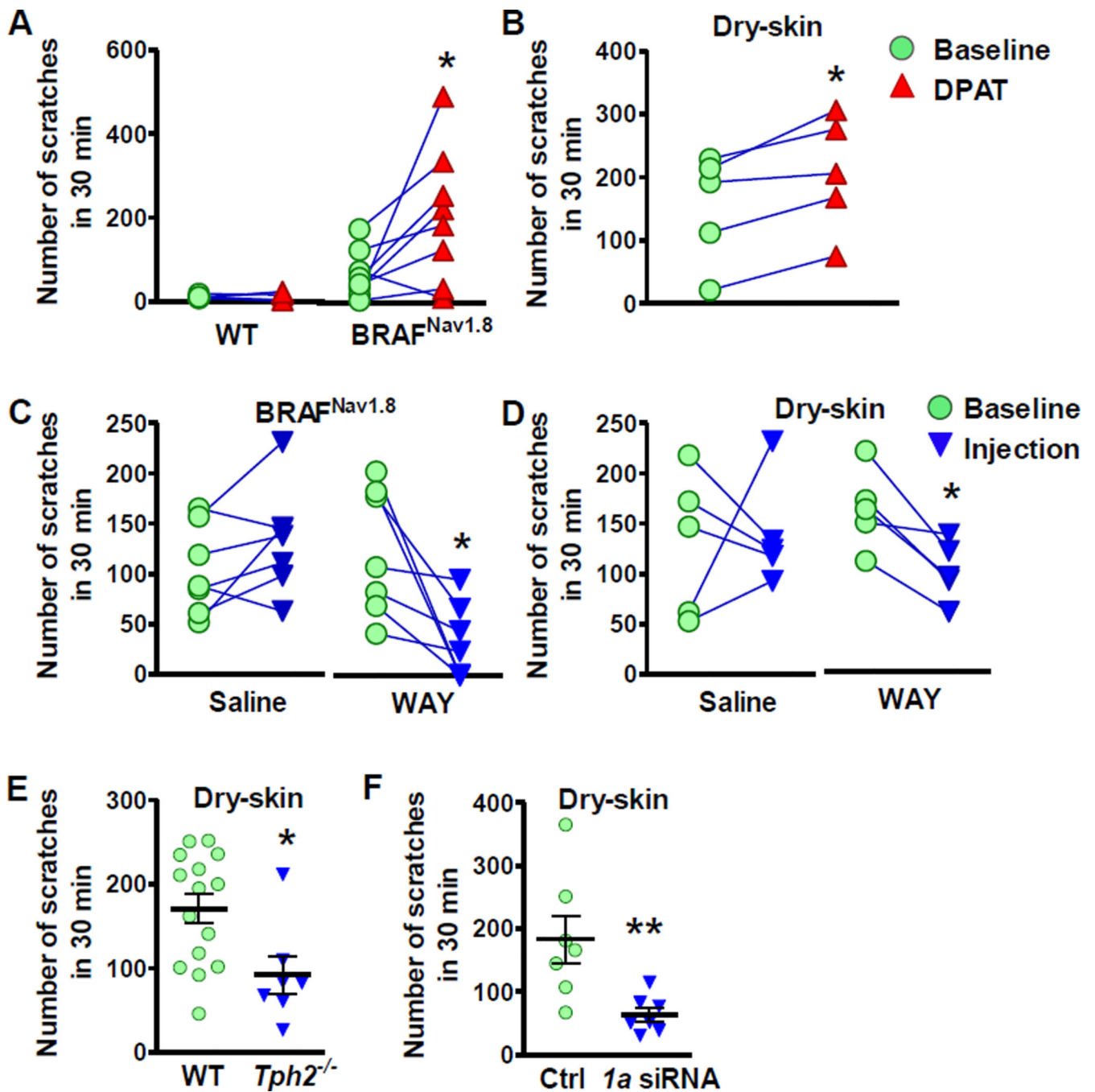
(D) Representative traces showing that a co-application of GRP and DPAT or 5-HT evoked membrane depolarization and AP firing in current-clamped GRPR<sup>+</sup> neurons. AP firing evoked by GRP+DPAT and GRP+5-HT ranged 0.167 to 3.5 Hz (n = 6) and 0.03 to 6 Hz (n = 17), respectively.

(E) Quantified data of A-D. GRP depolarized GRPR<sup>+</sup> neurons by 5.3 ± 0.3 mV (n = 8). DPAT (7.5 μM, blue) and 5-HT (40 μM, dark green) hyperpolarized GRPR<sup>+</sup> neurons by 9.4 ± 0.9 mV (n = 19) and 10.4 ± 1 mV (n = 13), respectively. In contrast, co-application of GRP +DPAT depolarized GRPR<sup>+</sup> neurons by 8.7 ± 1.4 mV (red) (n = 15). GRP+5-HT depolarized GRPR<sup>+</sup> neurons by 8.9 ± 1.5 mV (purple) (n = 35).

(F) Representative traces illustrate the effect of GRP (yellow), DPAT (blue), and GRP +DPAT (red) on membrane input resistance in current-clamped GRPR<sup>+</sup> neurons receiving negative current injections (-20 pA). Control (green): extracellular buffer only. For all

recordings, neuronal health was verified by observing action potentials in response to positive current injection under control conditions (black).

(G) GRP (yellow) increased the membrane input resistance from  $1,185 \pm 124 \text{ M}\Omega$  to  $1,350 \pm 150 \text{ M}\Omega$  ( $n = 8$ ), DPAT decreased the input resistance from  $1,311 \pm 168 \text{ M}\Omega$  to  $894 \pm 137 \text{ M}\Omega$  ( $n = 19$ ) and GRP + DPAT increased the input resistance from  $1,116 \pm 247 \text{ M}\Omega$  to  $1,616 \pm 226 \text{ M}\Omega$  ( $n = 15$ ),  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , paired  $t$ -test.



**Figure 7. Activation of 5-HT1A Facilitates Long-Lasting Itch Transmission**

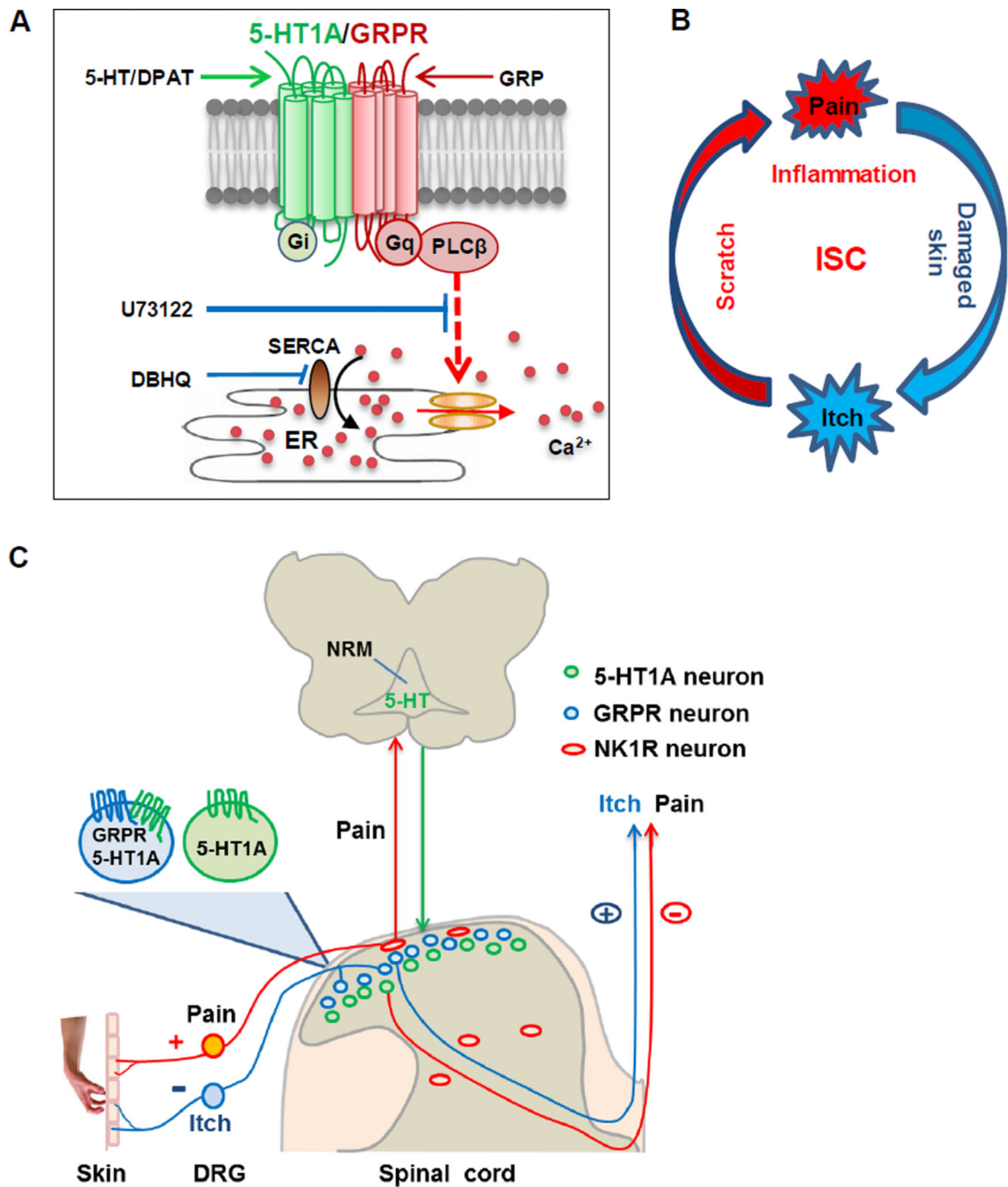
(A and B) Spontaneous scratching behaviors of BRAF<sup>Nav1.8</sup> mice (A) and dry-skin mice (B) were facilitated after DPAT injection.

(C and D) WAY (10 μg) suppressed spontaneous scratching behavior of BRAF<sup>Nav1.8</sup> mice (C) and dry-skin mice (D).

(E) *Tph2*<sup>-/-</sup> mice showed attenuated spontaneous scratching behavior under dry-skin condition compared to WT littermates.

(F) *Htr1a* siRNA treatment significantly attenuated spontaneous scratching behavior under dry-skin condition compared to control siRNA (Ctrl).

Error bars represent SEM.  $n = 5-15$ . \* $p < 0.05$ , paired  $t$  test in (A-D), unpaired  $t$  test in (E and F). See also Figures S7.



**Figure 8. Hypothetic Models Illustrating the Role of 5-HT/5-HT1A in the Itch-Scratch Cycle**  
 (A) Schematic showing signaling interactions between 5-HT1A and GRPR. Co-activation of 5-HT1A and GRPR amplifies G<sub>q</sub>-coupled PLC $\beta$ -dependent signaling pathway, resulting in the release of Ca<sup>2+</sup> from ER through SERCA pumps. ER: endoplasmic reticulum.  
 (B) Schematic that illustrates the vicious ISC. Scratching induces skin inflammation, which in turn induces more vicious itching attacks.  
 (C) Schematic of dual modulation of descending 5-HT signaling in pain and itch during the ISC. Scratching evokes inflammatory/mechanical pain, which results in the activation of 5-



HT neurons in NRM of the brainstem. Descending release of 5-HT acts on two distinct subpopulations of neurons: one expressing 5-HT1A alone (green cells) that inhibits nociceptive processing, while the other expressing both 5-HT1A and GRPR (blue cells) that facilitates pruriceptive transmission. NRM: nucleus raphe magnus.