Published in final edited form as:
Sci Signal. ; 4(181): ra45. doi:10.1126/scisignal.2001925.

The Distinct Roles of Two GPCRs, MrgprC11 and PAR2, in Itch and Hyperalgesia

Qin Liu[1,*], **Hao-Jui Weng**[1,*], **Kush N. Patel**[1,*], **Zongxiang Tang**[1,2], **Haihua Bai**[1,3], **Martin Steinhoff**[4,5], and **Xinzhong Dong**[1,6,†]

[1]Solomon H. Snyder Department of Neuroscience, Center for Sensory Biology, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA.

[2]Nanjing University of Chinese Medicine, 138 Xianlin Road, Nanjing 210046, China.

[3]Inner Mongolia University for the Nationalities, School of Life Science, 22 Huolinhe Street, Tongliao City 028043, China.

[4]Department of Dermatology, University of California, San Francisco, CA 94143, USA.

[5]Department of Surgery, University of California, San Francisco, CA 94143, USA.

[6]Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

Abstract

Itch has been defined as an unpleasant skin sensation that triggers the urge to scratch. Primary sensory dorsal root ganglia neurons detect itch stimuli through peripheral axons in the skin, playing an important role in generating itch. Itch is broadly categorized as histaminergic (sensitive to antihistamines) or nonhistaminergic. The peptide Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL) is an itch-inducing agent widely used to study histamine-independent itch. Here, we show that Mrgprs (Mas-related G protein–coupled receptors), particularly MrgprC11, rather than PAR2 (protease-activated receptor 2) as previously thought, mediate this type of itch. A shorter peptide, SLIGR, which specifically activates PAR2 but not MrgprC11, induced thermal pain hypersensitivity in mice but not a scratch response. Therefore, although both Mrgpr and PAR2 are SLIGRL-responsive G protein–coupled receptors present in dorsal root ganglia, each plays a specific role in mediating itch and pain.

†To whom correspondence should be addressed. xdong2@jhmi.edu.
*These authors contributed equally to this work.

SUPPLEMENTARY MATERIALS

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Fig. S1. Dose-response curves to SLIGRL for dissociated wild-type DRG neurons.
Fig. S2. SLIGRL fails to activate other mouse Mrgprs.
Fig. S3. The entire sequence of SLIGRL is required for the activation of MrgprC11.
Fig. S4. SLIGKV specifically activates human MrgprX2.
Fig. S5. RT-PCR analysis of DRG from WT (+/+), *PAR2*$^{-/-}$, and *Mrgpr-cluster*$\Delta^{-/-}$ mice (Mrgpr$^{-/-}$) mice for the abundance of mRNAs encoding PAR2, MrgprC11, and actin References.

Author contributions: Q.L., H.-J.W., K.N.P., Z.T., and H.B. performed the experiments and analyzed the data. M.S. provided PAR2 stable KNRK cells and PAR2 cDNA. Q.L., K.N.P., H.-J.W., and X.D. wrote the manuscript; M.S., K.N.P., and X.D. participated in revising the manuscript; and X.D. provided advice and guidance throughout.

Competing interests: The authors declare that they have no competing interests.

INTRODUCTION

The scratching response we associate with itch, or pruritoception, is the end result of a process initiated by the detection of itch-inducing stimuli. Primary sensory neurons in dorsal root ganglia (DRG) are responsible for the transduction of itch induced by pruritogens, which often act directly upon receptors within these fibers (1). Identification of cell surface receptors for pruritogens is essential for understanding itch signal transduction. Protease-activated receptors (PARs) are a family of four G protein–coupled receptors (GPCRs; denoted PAR1 to 4) whose N-terminal domains can be cleaved by serine proteases to unmask "tethered ligand" sequences (2). The tethered sequence subsequently self-activates the receptor to produce a downstream response. Certain PARs can be activated by synthetic peptides on the basis of the proteolytically cleaved tethered ligand sequence (2). PAR2 is activated by the proteases trypsin and tryptase and also selectively by the synthetic peptide Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL) (2), which is derived from its tethered ligand sequence. PAR2 is implicated in itch transduction because intradermal injections of trypsin and SLIGRL elicit scratching behavior in mice (3, 4), and skin application of either trypsin or PAR2 peptide agonist induces itch in humans (5, 6). In addition, this type of itch cannot be blocked by histamine H1 receptor antagonists, suggesting that it is histamine-independent (3, 4). However, it is unclear whether PAR2 is directly involved as a receptor that transduces the itch signal.

Mrgprs (Mas-related G protein–coupled receptors) encode a large family of GPCRs in the mouse genome, and many of them are present only in subsets of small-diameter sensory neurons in DRG and trigeminal ganglia (7). To determine the function of Mrgprs in vivo while overcoming the potential problem of gene redundancy, we generated a mouse line in which a cluster of 12 *Mrgpr* genes, including MrgprA3 and MrgprC11, was deleted (*Mrgpr-cluster*$\Delta^{-/-}$ mice) (8). Our analysis of *Mrgpr-cluster*$\Delta^{-/-}$ mice together with other evidence suggests that MrgprA3 and MrgprC11 function as itch receptors directly activated by the pruritogens chloroquine and bovine adrenal medulla peptide [BAM(8–22)], respectively. Chloroquine is an antimalarial drug that often evokes itch as a side effect in black Africans (9). BAM(8–22) is an endogenous opioid peptide that is derived from proenkephalin A (10). Because there are many itch-inducing reagents, it would be interesting to investigate whether the Mrgpr family can function as receptors for pruritogens other than chloroquine and BAM(8–22). Here, we report that MrgprC11, not PAR2, mediated SLIGRL-induced DRG neuron and scratching responses. In addition, a shorter peptide, SLIGR, which activates PAR2 but not MrgprC11, did not elicit scratching but induced thermal hyperalgesia in a PAR2-dependent manner.

RESULTS

SLIGRL-induced behavioral and neuronal responses are reduced in *Mrgpr-cluster*$\Delta^{-/-}$ mice

Previously, we have shown that *Mrgpr-cluster*$\Delta^{-/-}$ [knockout (KO)] mice exhibit reduced chloroquine- and BAM(8–22)-evoked scratching but normal histamine-dependent itch and acute pain sensitivity (8). To determine whether Mrgprs also play a role in SLIGRL-induced itch, we subcutaneously injected the peptide into the nape of the neck of *Mrgpr-cluster*$\Delta^{-/-}$ mice and wild-type littermates. The total scratching bouts during the first 30 min after injection were significantly decreased in mutant mice compared to wild-type littermates (Fig. 1, A and B).

Because Mrgprs are present only in DRG, the behavioral deficit seen in mutant mice can be attributed to a loss of SLIGRL responsiveness mediated by Mrgprs in primary sensory neurons. Indeed, application of 100 μM SLIGRL to cultured DRG neurons evoked a robust

increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in ~3% of cells from wild-type mice. In contrast, almost none of the neurons derived from *Mrgpr-cluster*$\Delta^{-/-}$ mice responded to SLIGRL (Fig. 1C). The percentage of DRG neurons responding to histamine was similar between wild-type and knockout groups (Fig. 1C). Dose responses to SLIGRL were also determined in wild-type DRG neurons, and the EC_{50} (median effective concentration) of the peptide was 51 μM (fig. S1). The small percentage of wild-type DRG neurons responding to the peptide correlated well with that of Mrgpr-containing cells in DRG (11). Furthermore, all SLIGRL-sensitive neurons also responded to BAM(8–22) (Fig. 1D). These data indicate that the SLIGRL-evoked $[Ca^{2+}]_i$ increases seen in wild-type DRG reflected specific activation of a subset of neurons and that this activation was Mrgpr-dependent. Using whole-cell patch-clamp recording, we also examined whether SLIGRL could directly induce action potentials in dissociated DRG neurons. In wild-type DRG, all SLIGRL-sensitive neurons, as identified by Ca^{2+} imaging, displayed a train of action potentials upon subsequent SLIGRL treatment and also responded to BAM(8–22) application (Fig. 1E).

MrgprC11 is activated directly by SLIGRL

The behavioral and neuronal loss-of-function phenotypes suggest that Mrgprs function as cell surface receptors for the PAR2 agonist peptide. To test this possibility directly, we examined whether Mrgprs could enable responses to SLIGRL in heterologous cells. We cloned each of the 12 *Mrgprs* deleted in *Mrgpr-cluster*$\Delta^{-/-}$ mice into a mammalian expression vector and transfected them individually into Chinese hamster ovary (CHO) cells because naïve CHO cells do not respond to SLIGRL. We also fused green fluorescent protein (GFP) in-frame to the C termini of the Mrgpr coding sequences to identify transfected cells and confirm proper membrane localization of the receptors. Our previous studies demonstrated that GFP does not alter the function of Mrgprs (7, 11). Increased $[Ca^{2+}]_i$ resulting from activation of the receptors was monitored by Ca^{2+} imaging. Of the 12 mouse Mrgprs, only expression of MrgprC11 elicited a response to SLIGRL in CHO cells (Fig. 2A). In contrast, other Mrgprs did not respond to the peptide; for example, MrgprA3-expressing CHO cells could be activated by chloroquine but not by SLIGRL (Fig. 2B and fig. S2). To determine the structural specificity of SLIGRL for MrgprC11 activation, we tested several SLIGRL derivatives, including SLIGRL without amidation ($-NH_2$), RL-NH_2, and the reverse peptide LRGILS-NH_2, on MrgprC11-expressing CHO cells (fig. S3). None of these derivatives induced significant calcium responses in cells, suggesting that the entire sequence of SLIGRL is required for MrgprC11 activation.

Because the percentage of mouse adult DRG neurons containing MrgprC11 (~3%) correlated well with SLIGRL sensitivity, we examined whether specific knockdown of MrgprC11 would abolish SLIGRL responsiveness. Wild-type DRG neurons failed to respond to SLIGRL after electroporation with small interfering RNA (siRNA) targeted against MrgprC11 (Fig. 2C), whereas MrgprA3 siRNA had no effect on SLIGRL sensitivity but abolished the chloroquine response (Fig. 2D). Finally, electroporation of MrgprC11 but not MrgprA3 complementary DNA (cDNA) into DRG neurons of *Mrgpr-cluster*$\Delta^{-/-}$ mice rendered them responsive to SLIGRL (Fig. 2, E and F). To determine the sensitivity of MrgprC11 to SLIGRL, we performed dose-response experiments in CHO cells. The EC_{50} of the peptide for MrgprC11 was 10.1 μM, which is comparable to that for PAR2 (4.8 μM) (Fig. 3, A and B), indicating that SLIGRL activates both receptors with similar potency. Together, these data suggest that MrgprC11 is a receptor that mediates SLIGRL-evoked scratching in mice.

The corresponding sequence of SLIGRL in human PAR2 is SLIGKV. Intracutaneous injection of SLIGKV peptide provokes itch in human subjects at the low millimolar range (5). To determine whether and which human MrgprXs can be activated by SLIGKV, we

transfected each of the four human MrgprXs (MrgprX1 to 4) individually into CHO cells. Only MrgprX2-expressing cells exhibited a response to SLIGKV as monitored by Ca^{2+} imaging, whereas MrgprX1-, X3-, or X4-expressing cells were insensitive to the peptide (fig. S4). Together, these data suggest that SLIGRL and SLIGKV can activate one mouse and one human Mrgpr member, respectively, with high specificity.

PAR2 is not required for SLIGRL- and trypsin-induced responses

Because SLIGRL can directly activate both PAR2 and MrgprC11 in heterologous cells, it is possible that PAR2 is also required for SLIGRL-induced itch. However, unlike *Mrgpr-cluster*$\Delta^{-/-}$ mice, *PAR2*$^{-/-}$ mice (fig. S5) showed similar scratching responses as wild-type mice to SLIGRL subcutaneously injected into the nape of the neck (Fig. 3C). Wild-type mice did not show a significant scratching response to RL-NH_2 or LRGILS-NH_2 compared to saline injection (Fig. 3C). In addition, the SLIGRL-induced calcium response in *PAR2*-deficient DRG neurons was similar to that of wild-type DRG neurons (Fig. 3D). Injection of trypsin into the nape of the neck in mice induces a scratching response (4). Because trypsin can cleave the N terminus of PAR2 to activate the receptor, and a PAR2 antagonist blocks trypsin-induced scratching (4), it has been proposed that PAR2 mediates trypsin-induced itch. Using *PAR2*$^{-/-}$ mice, we tested this possibility directly and found that the mutant mice scratched significantly more than did wild-type controls after trypsin injection (Fig. 3E). In addition, *Mrgpr-cluster*$\Delta^{-/-}$ mice showed comparable trypsin-induced scratching to controls (Fig. 3E).

A PAR2-specific peptide agonist induces thermal hyperalgesia but not scratching

To generate a PAR2-specific peptide agonist, we synthesized a new peptide, SLIGR-NH_2 (which lacked the C-terminal leucine of SLIGRL). Although SLIGR could still activate PAR2 with a slightly higher EC_{50} value (16.1 μM) than SLIGRL, it failed to activate MrgprC11 (Fig. 3, A and B). In addition, SLIGR did not induce increases in $[Ca^{2+}]_i$ as seen by subsequent SLIGRL treatment in wild-type DRG neurons (Fig. 4A). However, SLIGR can potentiate capsaicin-evoked calcium responses in wild-type DRG neurons and prevent desensitization from sequential capsaicin treatment (Fig. 4, B and C). Both SLIGR-mediated effects were significantly reduced in *PAR2*-deficient neurons (Fig. 4, B and C). This is consistent with previous findings that PAR2 enhances TRPV1 activity through the longer peptide SLIGRL (12). Unlike SLIGRL, subcutaneous injection of SLIGR into the nape of the neck did not induce significant scratching responses compared to saline injection (Fig. 3C). However, as previously reported for SLIGRL (13), plantar injection of SLIGR into the hindpaws produced strong thermal hyperalgesia (an increased pain response to noxious thermal stimuli) in wild-type mice, whereas *PAR2*$^{-/-}$ mice exhibited only a modest reduction of paw withdrawal latency to radiant heat after SLIGR injection (Fig. 4D). Together, these data suggest that the major function of PAR2 in DRG neurons is to induce hyperalgesia and that it plays a lesser role in itch signaling. Trypsin-induced thermal hyperalgesia was comparable among wild-type, Mrgpr knockout, and PAR2 knockout mice (Fig. 4E), suggesting that like trypsin-induced itch, trypsin-induced thermal hyperalgesia is independent of either MrgprC11 or PAR2.

DISCUSSION

SLIGRL is a widely used pruritogen for studying histamine-independent itch (14–18). Here, we show that Mrgprs (likely MrgprC11), not PAR2 as previously thought, are receptors that mediate this type of itch in mice. Our loss-of-function analyses indicate that Mrgprs, rather than PAR2, are required for behavioral and neuronal responses induced by SLIGRL. In line with these findings, SLIGRL directly activates only ~3 to 4% of total DRG neurons, which is consistent with the restricted presence of Mrgprs, particularly MrgprC11, in DRG (8, 11).

Moreover, all SLIGRL-sensitive neurons also respond to BAM(8–22), a specific agonist for MrgprC11 (8, 11). Like SLIGRL, BAM(8–22) induces an Mrgpr-dependent scratching response (8). These data further support the notion that Mrgprs are a family of GPCRs that play essential roles in itch responses by directly sensing different pruritogens.

Because MrgprC11 can be activated by many peptides with common C-terminal motifs, such as -RFamide, -RYamide, or -RYG (11), it is not completely surprising that SLIGRL is also an agonist forMrgprC11 among the 12 Mrgprs we tested. SLIGRL ends with -RLamide, and removing the leucine or amidation at the C terminus (for example, SLIGR or SLIGRL without NH_2) abolishes its ability to activate MrgprC11. These data suggest that MrgprC11 peptide agonists need arginine at the second to last position and amidation at their C termini. Although SLIGR can activate PAR2 at an EC_{50} comparable to that of SLIGRL as monitored by $[Ca^{2+}]_i$ increase in heterologous cells, it failed to directly induce any calcium response in wild-type DRG neurons where PAR2 is present. This discrepancy may be due to high PAR2 expression in the heterologous system, which results in coupling to the downstream calcium pathway. Our results with SLIGR, along with previous findings, suggest that PAR2 signaling in DRG neurons positively modulates TRPV1 activity mainly through protein kinase Cε (PKCε) and PKA pathways (19). Such modulation induces thermal hyperalgesia in a PAR2-dependent manner. Because SLIGR, which activates PAR2 but not MrgprC11, failed to evoke any scratching, it is unlikely that PAR2 plays a major role in itch by directly sensing the peptide in DRG neurons (Fig. 4F).Our results also suggest that trypsin-induced itch and thermal hyperalgesia are mediated by an unknown mechanism that is independent of PAR2 or Mrgprs, for example, through other PARs (Fig. 4F).

Although our data suggest that SLIGRL-induced scratching is mediated by MrgprC11, not PAR2, they do not diminish the roles of PAR2 in histamine-independent itch but broaden our knowledge of the complex roles of GPCRs in the physiology of pruritus. It is possible that itch induced by other PAR2 activators such as tryptase is mediated by PAR2 in humans (5). In addition, species differences in itch signaling may also explain the difference between our current findings in mice and activation of PAR2 observed in human pruritic responses. Further experiments using *PAR2*$^{-/-}$ mice, serine protease–deficient mice, and “humanized” PAR2 knock-in mice in skin disease models should address these issues. Previous studies have suggested that the activation mechanisms of PAR2 by peptides and proteases are likely to be different (20). Our study not only reveals a previously unappreciated role of MrgprC11 in peptide-induced itch but also emphasizes the differences between protease- and peptide-mediated itch signaling.

The role of MrgprC11 in SLIGRL-induced itch raises a possible interaction between PAR2 and MrgprC11, which may contribute to the physiological relevance of our finding. It is possible that soluble SLIGRL peptide is released from PAR2 by cleaving at the C terminus of SLIGRL sequence, which is a cleavage site for chymotrypsin on the basis of a computer program search for protease cleavage sites (21). Because both PAR2 and MrgprC11 are present in DRG neurons, it is conceivable that soluble SLIGRL can activate nearby MrgprC11. Indeed, intermolecular interaction between two PARs has been reported (22, 23). Future experiments are needed to test the potential interaction between PAR2 and MrgprC11 in vivo. Detailed genetic and molecular information about the roles of GPCRs in DRG neuron function will provide insight for developing novel anti-pain and anti-itch drugs.

MATERIALS AND METHODS

Knockout mice

The generation of *Mrgpr-cluster*Δ$^{-/-}$ mice was previously described (8). *PAR2*$^{-/-}$ mice were obtained from Jackson Laboratory and are in a C57BL/6 background.

Behavioral studies

All behavioral tests were performed with the experimenter blind to genotype. The mice were 2- to 3-month-old males (20 to 30 g) that had been backcrossed to C57BL/6 mice for at least 10 generations. Pruritic compounds (for example, SLIGRL, trypsin, and SLIGR) were subcutaneously injected into the nape of the neck after acclimatization, and scratching behavior was observed for 30 min. A bout of scratching was defined as continuous scratching movements with hindpaws directed at the area of the injection site. Scratching behavior was quantified by recording the number of scratching bouts at 5-min intervals over the 30-min observation period. Radiant heat (Hargreaves) was applied to the plantar surface of hindpaws before and after intraplantar injection of SLIGR (0.4 mg/ml, 8 μl) or trypsin (100 ng, 10 μl; Sigma) as previously described (13). Baseline response to a radiant heat stimulus was determined 2 days before SLIGR injection. The same radiant heat stimulus was applied to hindpaw 40 min after peptide injection. Thermal hyperalgesia is defined as a significant decrease in paw withdrawal latency to the radiant heat stimulus compared to baseline. All experiments were performed under the protocol approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine.

Culture of dissociated DRG neurons

DRG neurons from all spinal levels of 4-week-old mice were collected in cold DH10 [90% Dulbecco's modified Eagle's medium (DMEM)/F-12, 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml), Gibco] and treated with enzyme solution [dispase (5 mg/ml), collagenase type I (1 mg/ml) in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, Gibco] at 37°C. After trituration and centrifugation, cells were resuspended in DH10, plated on glass coverslips coated with poly-D-lysine (0.5 mg/ml) and laminin (10 μg/ml, Invitrogen), cultured in an incubator (95% O_2 and 5% CO_2) at 37°C, and used within 24 hours.

Electroporation of dissociated adult DRG neurons with Mrgpr expression constructs was carried out with a Mouse Neuron Nucleofector Kit (Amaxa Biosystems) following the manufacturer's instructions. Electroporated neurons were plated and cultured as described above.

Calcium imaging

Neurons or heterologous cells were loaded with fura 2–acetomethoxyl ester (Molecular Probes) for 30 min in the dark at room temperature or 45 min at 37°C, respectively. Cells were washed and then imaged at 340- and 380-nm excitation to detect intracellular free calcium. Calcium imaging assays were performed with the experimenter blind to genotype. Each experiment was done at least three times, and at least 100 cells (neurons or heterologous cells) were analyzed each time.

RNA interference

MrgprC11 or *MrgprA3* (0.175 nmol) on-target siRNA (Thermo Scientific) was electroporated into wild-type DRG neurons. After 3 days in culture, neurons were replated on glass coverslips for calcium imaging.

Whole-cell current-clamp recordings of cultured DRG neurons

Neurons plated on coverslips were transferred into a chamber with medium [the extracellular solution (ECS)] of the following composition: NaCl 140 mM, KCl 4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2 mM, Hepes 10 mM, and glucose 5 mM. pH was adjusted to 7.38 with NaOH. The intracellular pipette solution (ICS) contained KCl 135 mM, MgATP 3 mM, Na_2ATP 0.5 mM, $CaCl_2$ 1.1 mM, EGTA 2 mM, and glucose 5 mM. pH was adjusted to 7.38

with KOH, and osmolarity was adjusted to 300 mosM with sucrose. Chloroquine was stored at −20°C and diluted to 1 mM in ECS before use. Patch pipettes had resistances of 2 to 4 megohms. In current-clamp recordings, action potential measurements were performed with an Axon 700B amplifier and the pCLAMP 9.2 software package (Axon Instruments). Electrodes were pulled (Narishige, model PP-830) from borosilicate glass (WPI Inc.). Neurons were perfused with 1 mM SLIGRL or BAM(8–22) for 20 s. All experiments were performed at room temperature (~25°C).

Reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from various tissues with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was done with SuperScript III First-Strand (Invitrogen). Polymerase chain reaction conditions were as follows: PAR2: 94°C for 2 min, 35 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 72°C, and 7 min at 72°C; MrgprC11: 94°C for 2 min, 35 cycles of 15 s at 94°C, 30 s at 56°C, and 45 s at 72°C, and 6 min at 72°C. The MrgprC11- and PAR2-specific intron-spanning primers (to avoid genomic contamination) were as follows: MrgprC11-F, 5′-CAGCACAAGTCAGCTCCTCAA-3′, and MrgprC11-R, 5′-ATGCCCATGAGAAAGGACAGAACC-3′; PAR2-F, 5′-GGAAGGCTCAGTGAAGCTCGTGT-3′, and PAR2-R, 5′-TGCCAAGGAGACGCCAACGG-3′.

Data analysis

Data are presented as means ± SEM. Statistical comparisons were made with unpaired Student's *t* test and Wilcoxon rank-sum test, and differences were considered significant at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by an Alfred P. Sloan Neuroscience grant, a Whitehall Foundation grant, a Blaustein Pain Research Fund award, and grants from the NIH to X.D. (NS054791 and GM087369) and from the National Rosacea Society and West Havens Foundation to M.S. X.D. is an Early Career Scientist of the Howard Hughes Medical Institute. K.N.P. was supported by a Graduate Research Fellowship from the NSF.

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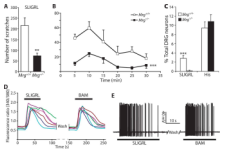

Fig. 1.

Mrgpr mediates SLIGRL-induced behavioral and neuronal responses. (**A** and **B**) *Mrgpr-cluster*$\Delta^{-/-}$ mice showed deficits in SLIGRL-induced itch. Total scratching bouts during the first 30 min after SLIGRL injection (50 μl, 2 mM) were significantly decreased in *Mrgpr-cluster*$\Delta^{-/-}$ mice ($n = 7$) compared to wild-type (WT) littermates ($n = 6$) (A). Time course of bouts of scratching at 5-min intervals (B). (**C**) Calcium imaging showed that ~3% of WT DRG neurons responded to SLIGRL (100 μM) with increased $[Ca^{2+}]_i$, whereas *Mrgpr-cluster*$\Delta^{-/-}$ DRG neurons failed to respond to the peptide (>600 neurons from three mice/genotype were analyzed; Wilcoxon rank-sum, $P < 0.005$). The percentage of *Mrgpr-cluster*$\Delta^{-/-}$ DRG neurons responding to histamine (His; 50 μM) was similar to that of WT neurons. (**D**) Representative traces from six different DRG neurons ($n = 11$) in calcium imaging assays, all of which also responded to BAM(8–22) (BAM, 2 μM). (**E**) In WT DRG neurons, all SLIGRL-sensitive neurons (as identified by calcium imaging, $n = 6$) displayed a train of action potentials evoked by subsequent SLIGRL treatment. All of them also generated action potentials in response to BAM(8–22) treatment. Data are presented as means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; $^{\#}P < 0.001$; two-tailed unpaired *t* test, Wilcoxon rank-sum test, or two-way analysis of variance (ANOVA).

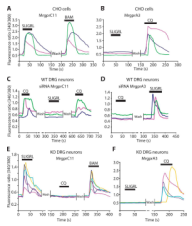

Fig. 2.

SLIGRL specifically activates MrgprC11. (**A** and **B**) Representative traces from three different CHO cells transfected with different Mrgprs in calcium imaging assays. MrgprC11-expressing CHO cells (n = 150) responded to SLIGRL (10 μM) and BAM(8–22) (2 μM) (A). MrgprA3-expressing cells (n = 155) responded to chloroquine (CQ; 100 μM) but not SLIGRL (B). (**C** and **D**) Representative traces in calcium imaging assays of three different WT DRG neurons electroporated with siRNAs. SLIGRL-induced (100 μM) increase in $[Ca^{2+}]_i$ was lost in WT neurons electroporated with *MrgprC11* siRNA, although these neurons were still sensitive to an MrgprA3 agonist, CQ (1 mM) (n = 7 CQ-sensitive neurons) (C). As a control, SLIGRL responsiveness in WT neurons electroporated with *MrgprA3* siRNA remained intact (n = 10 SLIGRL-sensitive neurons), but electroporation of *MrgprA3* siRNA completely abolished CQ sensitivity (D). (**E**) All *MrgprC11*-expressing *Mrgpr-cluster*$\Delta^{-/-}$ neurons (n = 66; GFP^+ neurons) showed increased $[Ca^{2+}]_i$ in response to SLIGRL (100 μM) and BAM(8–22) (2 μM) but not to CQ (1 mM). (**F**) All *MrgprA3*-electroporated mutant neurons (n = 72) showed a strong response to CQ (1 mM) but not to SLIGRL (100 μM).

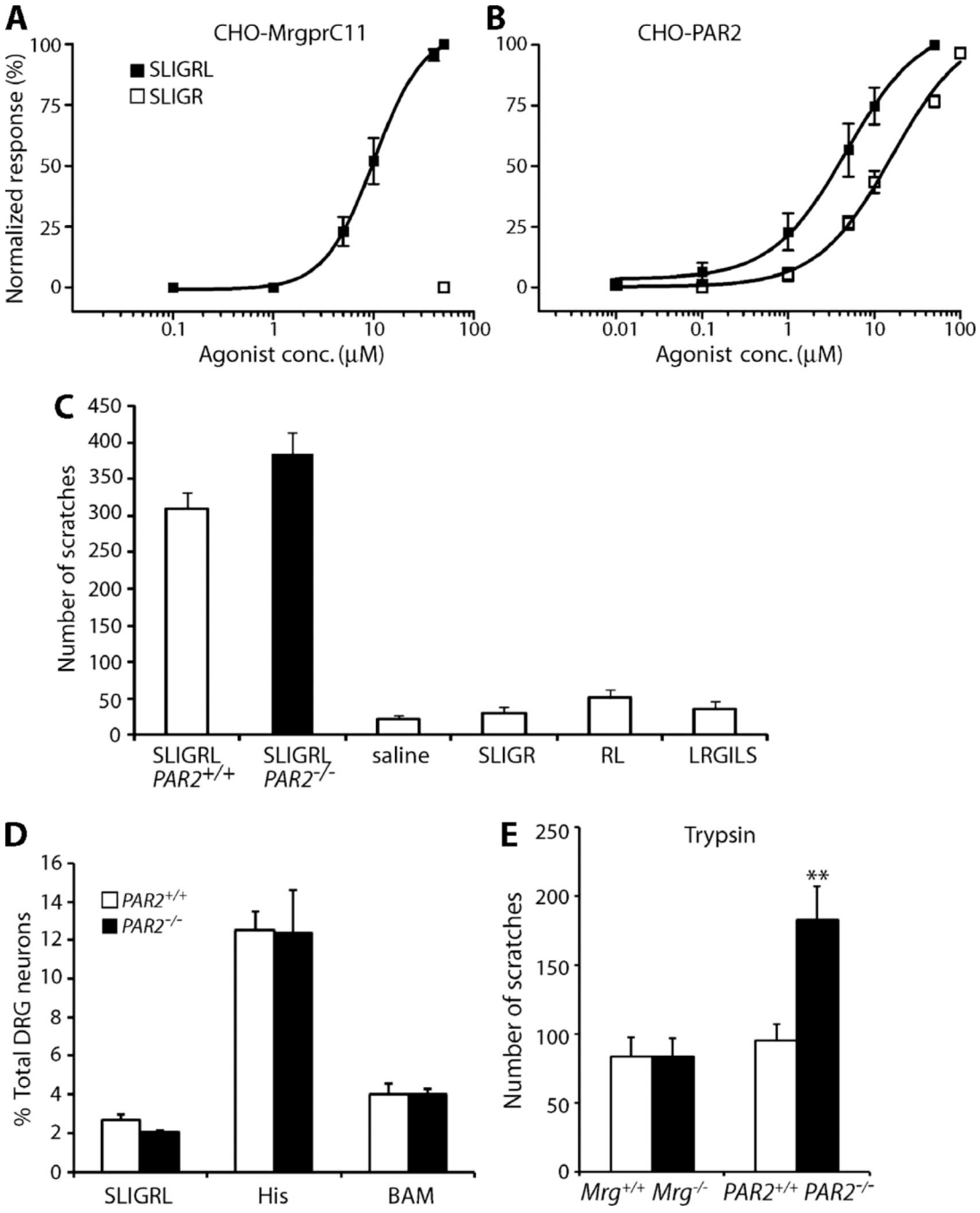

Fig. 3.

PAR2 plays a minimal role in SLIGRL-induced itch. (**A** and **B**) Dose-response curves to SLIGRL and SLIGR for MrgprC11 (A) and PAR2 (B) expressed in CHO cells. SLIGR activates PAR2 but not MrgprC11. Each data point represents the mean ± SEM of at least three independent experiments and at least 100 cells were analyzed each time. Calcium responses at each peptide concentration were normalized to the maximal response elicited subsequently. (**C**) PAR knockout mice ($PAR2^{-/-}$, $n = 9$; black bar) showed a mild but nonsignificant increase in SLIGRL-induced itch compared with WT mice ($n = 9$). Injection of 50 μl of saline, 2.0 mM each SLIGR, RL-NH_2, or LRGILS-NH_2 did not evoke significant scratching in WT mice ($n = 5$, 9, 5, and 5, respectively). (**D**) The percentages of $PAR2^{-/-}$

DRG neurons responding to SLIGRL (100 μM), histamine (50 μM), and BAM(8–22) (2 μM) were similar to those of WT neurons. (**E**) Trypsin-induced scratching (200 μg, 50 μl) was significantly stronger in *PAR2*$^{-/-}$ mice (n = 6) than WT mice (n = 6). *Mrgpr-cluster*$\Delta^{-/-}$ mice (n = 8) and their WT littermates (left; n = 6) exhibited similar scratching responses to trypsin.

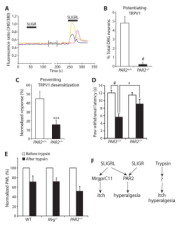

Fig. 4.

PAR2 mediates thermal hyperalgesia–induced SLIGR. (**A**) Representative traces from three different WT DRG neurons in calcium imaging assays (n = 722). Unlike SLIGRL, SLIGR failed to induce an increase in $[Ca^{2+}]_i$. (**B** and **C**) WT DRG neurons (n = 180) were stimulated with capsaicin (15 nM) followed by SLIGR (100 μM) and capsaicin (15 nM) to study the effect of PAR2 on TRPV1 activity as monitored by calcium imaging. About 5% of WT ($PAR2^{+/+}$) DRG neurons that did not initially respond to capsaicin became capsaicin-sensitive after SLIGR treatment, a potentiating effect that was significantly reduced in $PAR2^{-/-}$ neurons compared to WT cells (B). WT DRG neurons responding to the initial capsaicin treatment (n = 33) exhibited significantly less desensitization to the second capsaicin application after SLIGR treatment than $PAR2^{-/-}$ neurons (n = 62) (C). (**D**) Paw withdrawal latency in response to radiant heat (the Hargreaves test) was determined before (open bars) and after (black bars) intraplantar injection of SLIGR (0.4 mg/ml, 8 μl) into WT (n = 10) and $PAR2^{-/-}$ (n = 10) mouse hindpaws. Thermal hyperalgesia (as assessed by reduction in latency) induced by the peptide was significantly decreased in $PAR2^{-/-}$ mice compared with WT mice. (**E**) Paw withdrawal latency (PWL) in response to radiant heat (measured by the Hargreaves test) was determined before (open bars) and after (black bars) intraplantar injection of trypsin (100 ng, 10 μl) into WT (n = 7), *Mrgpr-cluster*$\Delta^{-/-}$ (n = 8), and $PAR2^{-/-}$ (n =10) mouse hindpaws. Thermal hyperalgesia (reduction in latency) induced by trypsin was comparable among the three groups. (**F**) Comparison of the roles of Mrgpr and PAR2 in itch and hyperalgesia.