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Genetic enhancement of calcitonin gene-related peptide-induced central sensitization to mechanical stimuli in mice

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Abstract

Calcitonin gene-related peptide (CGRP) is a key player in migraine. To address the role of CGRP in mechanical allodynia, which is a common feature of migraine, we used CGRP-sensitized transgenic mice. These mice have elevated nervous system expression of the human receptor activity-modifying protein-1 (hRAMP1) subunit of the CGRP receptor. Under baseline conditions, the nestin/hRAMP1 mice and control littermates had similar hindpaw withdrawal thresholds to von Frey filaments. The effect of CGRP was tested using a filament that elicited a withdrawal response on 20% of its presentations. Following intrathecal injection of 1 nmol CGRP in the nestin/hRAMP1 mice, the response frequency was 80% within 30 min. The antagonist CGRP(8-37) blocked the increased response. In control littermates, a five-fold higher dose of CGRP was required to elicit a similar response. In contrast to intrathecal injection, peripheral CGRP did not increase the mechanical responses. Intraplantar injection of capsaicin was used to test the efficacy of endogenous CGRP. Capsaicin increased mechanical responses in the nestin/hRAMP1 and control mice, although a higher dose was required in controls. In contrast to control mice, there was also a contralateral paw response in nestin/hRAMP1 mice, which is consistent with central sensitization. **PERSPECTIVE:** In this study we show central CGRP-induced mechanical allodynia that is enhanced by overexpression of RAMP1 in nervous system. These data suggest that hypersensitivity to CGRP could be a potential mechanism underlying central sensitization in migraine and point to CGRP receptor antagonists as a possible therapy for other pain disorders.

Keywords

CGRP; RAMP1; mechanical allodynia; nociception; capsaicin; central sensitization

INTRODUCTION

The neuropeptide calcitonin gene-related peptide (CGRP) is now recognized to be a key player in migraine based on three lines of evidence. Injection of CGRP can induce severe headache in migraineurs 31, CGRP levels are elevated during evoked migraine 28 and have been reported elevated during spontaneous migraine 23,25,51. While a recent well-controlled study has questioned whether CGRP levels are increased during migraine 51, this unresolved question

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may be explained by a report that basal CGRP levels are already elevated in migraineurs 3. Finally, CGRP receptor antagonists have proven effective in clinical trials 27,41. A role for CGRP in migraine is consistent with its actions in vasodilation and neurogenic inflammation 8,9,37,53, and the relay of nociceptive signals and central sensitization 1,7.

While it is generally accepted that CGRP plays an important modulatory role in nociception 7,17,20,21,40,45,47–49, the mechanisms and sites of action by which it contributes to central sensitization are not well established. Most studies have concluded that CGRP-induced nociception is centrally mediated on post-synaptic spinal neurons, however, a peripheral role has also been suggested. CGRP injections in the paw increased the response to an innocuous mechanical stimulus 38 and peripheral CGRP administration reestablished mechanical allodynia secondary to capsaicin injection after dorsal rhizotomy 32. Furthermore, elevated CGRP alone is unlikely to be responsible for migraine pain because injection of CGRP into non-migraineurs did not generate migraine-like symptoms 42. Thus, the mechanisms by which CGRP might contribute to mechanical sensitivity in general, and to migraine in particular, remain unclear.

We have speculated that migraine may involve an increased sensitivity to CGRP-mediated modulation of nociceptive pathways 46. One possible means for elevated sensitivity would be an increased number of CGRP receptors. The CGRP receptor is an unusual G protein coupled protein that has an obligate requirement for a small transmembrane subunit called receptor activity modifying protein-1 (RAMP1) and is associated with an intracellular receptor coupling protein (RCP) (Fig. 1A) 43. RAMP1 acts as a chaperone to transport the calcitonin-like receptor (CLR) to the cell surface and is required for binding of CGRP to the CLR/RAMP1 complex 26. Using viral mediated gene transfer, we previously demonstrated that RAMP1 is functionally rate limiting for CGRP receptor activation in cultured vascular smooth muscle and trigeminal ganglion neurons 54,55. A cre-mediated activation strategy was used to generate transgenic mice that overexpress human RAMP1 (hRAMP1) in the nervous system. As seen with the culture studies, expression of hRAMP1 sensitized the mice to CGRP, with an increase in CGRP-induced plasma extravasation 55.

To test whether there might be a link between sensitivity to CGRP and sensitivity to touch, we examined mechanical allodynia in the nestin/hRAMP1 mice. Mechanical allodynia is perception of a normally innocuous mechanical stimulus as painful. Migraineurs experience sensitization to cutaneous stimuli, including mechanical allodynia in 40–50% of cases 15,24,34,36. While allodynia in migraineurs is more predominant in the facial region, 36% of the patients reporting allodynia also reported extracephalic allodynia. Allodynia appears as the migraine progresses and has been associated with central sensitization 10,12,14,30.

In this proof of principle study, we have chosen to use a well-established hindpaw withdrawal assay for CGRP-induced mechanical allodynia. This assay is especially appropriate for the nestin/hRAMP1 mouse model because hRAMP1 is expressed throughout the central and peripheral nervous systems. We provide evidence that CGRP acts in the central nervous system to confer sensitivity to mechanical stimuli. This sensitization is greatly enhanced in mice with elevated nervous system expression of hRAMP1. These findings suggest that increased sensitivity to CGRP may play a role in development of the central sensitization associated with migraine.

MATERIALS AND METHODS

Transgenic mice

Generation of the nestin/hRAMP1 double transgenic mice has been described 55. Both males and females ages 10 to 45 weeks were used. Food and water were provided ad libitum. The

double transgenic nestin/hRAMP1 mice were generated by crossing mice with a hybrid β -actin/CMV promoter and stop sequences flanked by loxP sites upstream of the hRAMP1 cDNA (generated by University of Iowa Transgenic Animal Facility) with nestin-cre mice (originally from Jackson Laboratories, Maine, USA). Cre recombinase is required for removal of the translational stop sequence and a polyadenylation signal that otherwise prevent expression of hRAMP1. The nestin-cre mice express cre by embryonic day 11, primarily in progenitor cells of the central and peripheral nervous system and isolated cells in the heart and kidney 19. All procedures were approved by the University of Iowa Animal Care and Use committee and performed under the guiding principles of the Society for Neuroscience and American Physiological Society. Two strains of nestin cre mice with the same transgene, but on different genetic backgrounds were used. Unless otherwise stated, all studies were done using mice with nestin cre on a mixed C57Bl/6J and 129SVE background.

Tissue Extraction and Q-PCR

Total RNA was isolated from lumbar spinal cord using Trizol reagent (Invitrogen), purified using the RNeasy kit (Qiagen), and RNA concentration was determined by spectrometry. DNA contamination was removed from 1 μ g by digestion with Amp Grade DNase I (Invitrogen) in 10 μ l as recommended. Reverse transcription was performed using the Taqman RT-PCR mix (Applied Biosystems) with 0.5 μ g RNA, 1X RT buffer, 5.5 mM MgCl₂, 0.5 mM dNTP, 4 units RNase inhibitor, 2.5 μ M random hexamers, 12.5 units Multiscribe reverse transcriptase in 10 μ l at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. Q-PCR was performed using 50 ng cDNA, 670 nM each primer, 0.026 units Imolase DNA polymerase (Bioline), 1X Immo Buffer, 6 mM MgCl₂, 400 μ M each dNTP, 5% DMSO, 0.005% SYBR Green (Invitrogen), 0.1 mM fluorescein (EMD Biosciences), 0.02% tween-20 in 15 μ l. The cycle conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and annealing/extension at 60°C for 30 s and 72°C for 45 s. Reactions were performed in triplicate and analyzed using a Bio-Rad MY-IQ thermocycler. At the end of amplification, a thermal melt curve was generated. Samples that did not yield a homogenous melt curve were not included. The primers for mRAMP1, hRAMP1, and mGAPDH have been described 55. The mRAMP1, hRAMP1, and GAPDH Ct values were converted to absolute copy numbers using standard curves generated with serial dilutions of pGEM-QmRAMP1, pbsCX1-LEL-hRAMP1, and pQmGAPDH plasmids, respectively.

Drugs

CGRP and CGRP(8-37) (Sigma) were dissolved in phosphate buffered saline (PBS). CGRP or PBS vehicle were injected intrathecally (1 or 5 nmol in 10 μ l), subcutaneously in the subscapular region (2.6 nmol/kg) or in the plantar surface of the hindpaw of the mice (1.3 nmol in 10 μ l) under brief 3–5% isoflurane anesthesia. Intrathecal injections were done using a 30 g needle attached to a Hamilton syringe between L4 and L5 vertebrae. CGRP(8-37) (5 nmol in 10 μ l) was injected intrathecally in the same solution with CGRP or vehicle. Capsaicin (Sigma) was dissolved in PBS with 5% Tween 80 and 5% ethanol. Capsaicin (0.01% and 0.001%, 10 μ l) or vehicle was injected in one plantar surface of the hindpaw under brief isoflurane anesthesia.

Paw Withdrawal Assay

In all experiments the investigator was blinded to the treatment and the genotype of the mice. For baseline experiments, mice were acclimated for 3 h per day for 4–5 days in the test chambers on top of a soft wire mesh floor. On the day of the test, a set of six von Frey filaments was used in increasing order from E3.22 to J4.17. Filaments were labeled alphabetically (from A to J) in order of increasing applied force. The set of filaments applied, from E to J, correspond to increasing forces of 1.5 (E), 3 (F), 5 (G), 9.8 (H), 13.7 (I) and 19.6 (J) mNewtons or 0.16, 0.4,

0.6, 1.0, 1.4 and 2.0 g, respectively. Every filament was applied five times to each hindpaw and the response was measured as the percentage of paw withdrawal responses to each filament. No significant difference was found between the responses of right and left hindpaws. Therefore, the responses of right and left hindpaws were averaged within each animal to yield a single value for that mouse.

For the CGRP and capsaicin experiments, mice were acclimated to the room for 3 h per day for 3 days prior to the experiment. On the day of the experiment, mice were acclimated to the test chamber on top of a soft wire mesh floor for 1 h. Baseline measurements for the F filaments were taken and the animals were briefly removed from the chambers for the drug injection. After CGRP administration the filament was re-applied 30, 60 and 90 min after intrathecal injections or 5, 15, 30 and 60 min after intraplantar injections. For the intrathecal experiments, right and left hindpaw responses were averaged. No significant difference was found between the responses of right and left hindpaws in the intrathecally treated animals. For the intraplantar experiments, ipsilateral and contralateral sides were analyzed separately. Following capsaicin injection, there was a spontaneous licking response that lasted less than 15 min. The E or F filament was applied at 15, 30, 45 and 60 min after intraplantar injection of capsaicin. Withdrawal frequencies for the ipsilateral and contralateral hindpaws were analyzed separately.

Nocifensive behavior was also measured after injection of 0.01% capsaicin. The number of times that the mouse licked the injected hindpaw was counted in 5 min blocks during the 15 min immediately following intraplantar injection.

Statistical analyses

For the analysis of the CGRP mRNA levels, a paired Student t test was performed. Responses to repeated applications of von Frey filaments were analyzed using a two-way analysis of variance (ANOVA) for repeated measures. If overall significance was obtained, an all pairwise multiple comparison procedure (Student-Newman-Keuls method) was performed to study the difference between time points, between groups and between groups at different time points in case of interaction. Data are expressed as mean $\pm$ SEM.

RESULTS

Spinal Cord RAMP1 mRNA Levels

The nestin/hRAMP1 mice selectively express the hRAMP1 transgene throughout the peripheral and central nervous system ⁵⁵. For this study, we further examined the lumbar spinal cord. Mouse and human RAMP1 mRNA was measured because, unfortunately, RAMP1 protein levels cannot be reliably determined due to difficulties with available antibodies ⁵⁵. There is about a 2-fold greater level of total RAMP1 mRNA in the lumbar spinal cord of the nestin/hRAMP1 mice than in littermate controls (Fig. 1B). This agrees with the previously reported ~2-fold greater level of total RAMP1 mRNA in the brain and trigeminal ganglia ⁵⁵. Mouse RAMP1 mRNA levels were essentially the same in both groups of mice. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control for mRNA quantity, integrity, and normalization.

Similar Baseline Mechanical Nociception in nestin/hRAMP1 Mice and Littermate Controls

The response to mechanical stimulation was measured using a series of von Frey filaments of increasing force. Each filament was tested five times in rapid succession on one hindpaw, followed by testing on the other hindpaw, to calculate the response frequency to the filament. The stimulus-response relationship for mechanical stimulation was the same in nestin/hRAMP1 and littermate controls (Fig. 2).

Central CGRP Injection Increased Mechanical Nociception

To test the effect of CGRP on mechanical nociception, we selected a mild stimulus paradigm. The F filament was chosen because it triggered paw withdrawal in only 10–20% of the trials. Intrathecal injection of 1 nmol CGRP into the lumbar spinal region did not elicit a significant increase in frequency of paw withdrawal by control mice (Fig. 3A). In contrast, there was a marked and sustained increase in paw withdrawal frequency following intrathecal injection of 1 nmol CGRP in the nestin/hRAMP1 mice (Fig. 3B). In the nestin/hRAMP1 mice, response frequency was increased to 80% at 30 min after injection of 1 nmol CGRP compared to 30% after vehicle injection. By 60 min, the response had reached 100% with the nestin/hRAMP1 mice. The increased paw withdrawal frequency was blocked by co-injection with CGRP(8-37) (Fig. 3B, C). Interestingly, injection of antagonist alone appeared to lower the response frequency in control animals (data not shown), suggesting that repeated mechanical stimulation could evoke release of endogenous CGRP.

To test if a differential sensitivity to CGRP contributed to this difference between the control and nestin/hRAMP1 mice, we tested a higher dose of CGRP (5 nmol) in the control mice. Thirty min after intrathecal injection of 5 nmol CGRP, the response frequency was increased to 70%, compared to about 25% after vehicle injection. By 60 min, the response reached a plateau at 80% (Fig. 3A).

During the course of these experiments, we realized that the nestin-cre mice strain had not been maintained on a pure C57Bl/6 background, but had become out-bred with littermates from the double transgenic crosses (C57Bl/6 and SJL) and a 129SVE colony. To rule out contributions from the mixed genotype of the nestin-cre mice, we obtained mice with nestin-cre in the original C57Bl/6J background (B6.Cg-Tg(Nes-cre)1Kln/J, Stock No. 003771, Jackson Laboratory). As seen with the mixed genotype nestin-cre mice, intrathecal CGRP (1 nmol) enhanced the mechanical response of transgenic mice, but not controls, 30 min after injection (data not shown).

Peripheral CGRP Injection Did Not Increase Mechanical Nociception

In contrast to central administration, intraplantar injection of 1.3 nmol CGRP did not increase the response frequency to the F filament in either hRAMP1 (Fig. 4A) or control mice (Fig. 4B). The responses were also similar to the frequencies observed in the contralateral paw (Fig. 4C, D). This dose of CGRP has been reported to produce mechanical allodynia in mice 38. We also did not see any effect on paw withdrawal frequencies at 30, 60 and 90 min following subcutaneous injection of 2.6 nmol/kg into the subscapular region (data not shown). This site of administration and dose of CGRP has been reported to be sufficient for systemic vasodilation 44.

Capsaicin Increased Mechanical Nociception in Both the Ipsilateral and Contralateral Paw in nestin/hRAMP1 Mice

We next tested the effects of an agent known to increase release of endogenous CGRP, the vanilloid capsaicin. Local intraplantar injection of 0.01% capsaicin increased the frequency of paw withdrawal to the F filament in both nestin/hRAMP1 and control littermates (Fig. 5A). The response in the ipsilateral paw was close to 100% in both groups of animals. This strong response would not allow us to observe a higher response in one of the groups. Interestingly, there was also an increased response observed with the contralateral paw of the nestin/hRAMP1, but not control, mice (Fig. 5B). The degree of increase seen with control mice is similar to that normally observed following vehicle injection due to the repeated testing (see Fig. 4). There was no difference in the number of lickings (Control: 47 ± 14 , nestin/hRAMP1: 67 ± 21), or in the duration of the nocifensive behavior during the 15 min after capsaicin injection. Given the robust response to the F filament, we then tested a lower force filament

(E, which produced a baseline response near 0%) and a lower dose of capsaicin. The nestin/hRAMP1 and control mice showed the same pattern of response to 0.01% capsaicin with the E filament as with the F filament (data not shown). On the other hand, intraplantar injection of 0.001% capsaicin increased the response to the F filament with both the ipsilateral (Fig. 5C) and contralateral paws (Fig. 5D) in only the nestin/hRAMP1 mice, not the control littermates. The increase from repeated testing seen with the control mice was comparable to that seen following injection of vehicle (see Fig. 4).

DISCUSSION

A recognized symptom of migraine is heightened sensitivity to stimuli, including mechanical allodynia. The perception of touch as a painful stimulus is reported by nearly half of migraineurs 34,36. Mechanical allodynia is even more frequent in patients with transformed, chronic migraine than episodic migraine, it correlates with the duration of the migraine 6,33, and it is a good predictor that a migraine has progressed to a point of resistance to triptan drugs 11,13. For these reasons alone, the mechanisms underlying allodynia are of particular interest.

In this study, we have shown that intrathecal injection of CGRP increases sensitivity to mechanical stimuli in mice. Importantly, sensitivity to the allodynic effects of CGRP was further enhanced in mice with elevated expression of the hRAMP1 subunit of the CGRP receptor. The two-fold elevation in total RAMP1 mRNA in the spinal cord is consistent with our previous measurements from brain and trigeminal ganglia 55. It is striking that at the lower dose of CGRP, there was up to a 100% response rate with the nestin/hRAMP1 mice, but little or no CGRP-induced response with the control mice. Thus, RAMP1 appears to be functionally rate-limiting for CGRP-induced mechanical nociception, which agrees with its enhancement of receptor activation of intracellular signaling pathways 54,55.

Our data indicate that CGRP is acting centrally to sensitize the mice to mechanical stimuli. In contrast to the positive results with intrathecal administration, peripheral injection of CGRP did not alter the mechanical withdrawal threshold. In addition, the observation of contralateral paw sensitivity in the nestin/hRAMP1 mice following capsaicin treatment is consistent with a central action of CGRP release. A central site of action agrees with most of the literature. Injection of CGRP into the trigeminal nucleus of the brainstem elicits a cardiovascular response that is similar to painful stimuli 2,5. Likewise, intrathecal injection of CGRP increased the response to a mechanical stimulus 38, whereas administration of a CGRP receptor antagonist in the spinal cord dorsal horn partially reversed thermal and mechanical allodynia secondary to intradermal capsaicin injection and in a model of chronic pain 4,48–50,52. A role for CGRP in spinal synaptic plasticity in a model of arthritic pain has also been reported 7. Furthermore, a central site of action is supported by the prevalence of CGRP receptors on postsynaptic second order spinal cord neurons 16,35. However, it has been reported that local injection of CGRP in the paw can induce mechanical allodynia in mice 38. The reason for this discrepancy is not known, but perhaps could be due to differences in experimental design and/or strain background 39.

Peripheral CGRP is likely to play other important roles. Peripheral, as well as central, CGRP has been reported to modulate nociceptive input via central pathways 18. In the periphery, there are CGRP receptors on mast cells, the vasculature, and a relatively low level on dorsal root ganglion cell bodies and peripheral terminals 16,35. At the periphery, it seems likely that the primary role of CGRP may be to stimulate vasodilation and release of bradykinin and cytokines from mast cells and neuropeptides from sensory terminals 32,55. This would increase neurogenic inflammation that would further sensitize the nociceptors.

An important point is that CGRP increased paw withdrawal frequency not only in the nestin/hRAMP1 mice, but also in control mice, albeit a higher amount of CGRP was needed to show a similar effect. This indicates that overexpression of the hRAMP1 subunit sensitized mice to nociceptive signaling that is a normal function of CGRP. The observation that the CGRP(8-37) antagonist appeared to reduce the learned sensitivity to touch suggests that sequential application of a mechanical stimulus releases endogenous CGRP, which results in a higher response to the next stimulus. In this regard, we demonstrated that endogenous levels of CGRP were sufficient to activate the allodynic response in the nestin/hRAMP1 mice. This was shown by using capsaicin to activate the TRPV1 ion channels on nociceptive neurons, which is known to trigger the release of CGRP. Intrathecal injection of a CGRP antagonist blocked capsaicin-induced secondary mechanical allodynia in rats 48–50, suggesting that CGRP is involved in central sensitization induced by capsaicin. Centrally-mediated sensitization of primary afferent nociceptors by intradermal capsaicin can also involve peripheral release of CGRP 32.

The findings of this study demonstrate that elevation of RAMP1 provides a mechanism for enhancement of CGRP-induced central sensitization. RAMP1 is regulated by a number of stimuli 26. It is especially intriguing that peripheral inflammation can lead to changes in CGRP binding sites in the dorsal horn of the spinal cord 22,29. Given the growing evidence for a causal role for CGRP in migraine and the likelihood of neurogenic inflammation during migraine, it seems likely that the enhanced mechanical allodynia seen with this mouse model may reflect a role for CGRP and RAMP1 in central sensitization in migraine. Furthermore, a CGRP antagonist has been shown to abolish mechanical and thermal sensitivity in an animal model of central neuropathic pain 4, and a role for CGRP has been documented in other chronic pain conditions, such as arthritis 7. Given the role that CGRP plays in central sensitization, it is tempting to speculate that CGRP receptor antagonists may block sensitization, not only in migraine, but also in other pain disorders.

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References

1. Adwanikar H, Ji G, Li W, Doods H, Willis WD, Neugebauer V. Spinal CGRP1 receptors contribute to supraspinally organized pain behavior and pain-related sensitization of amygdala neurons. *Pain* 2007;132:53–66. [PubMed: 17335972]
2. Allen GV, Barbrick B, Esser MJ. Trigeminal-parabrachial connections: possible pathway for nociception-induced cardiovascular reflex responses. *Brain Res* 1996;715:125–135. [PubMed: 8739631]
3. Ashina M, Bendtsen L, Jensen R, Schifter S, Olesen J. Evidence for increased plasma levels of calcitonin gene-related peptide in migraine outside of attacks. *Pain* 2000;86:133–138. [PubMed: 10779670]
4. Bennett AD, Chastain KM, Hulsebosch CE. Alleviation of mechanical and thermal allodynia by CGRP (8-37) in a rodent model of chronic central pain. *Pain* 2000;86:163–175. [PubMed: 10779673]
5. Bereiter DA, Benetti AP. Microinjections of calcitonin gene-related peptide within the trigeminal subnucleus caudalis of the cat affects adrenal and autonomic function. *Brain Res* 1991;558:53–62. [PubMed: 1718570]
6. Bigal ME, Ashina S, Burstein R, Reed ML, Buse D, Serrano D, Lipton RB. Prevalence and characteristics of allodynia in headache sufferers: a population study. *Neurology* 2008;70:1525–1533. [PubMed: 18427069]
7. Bird GC, Han JS, Fu Y, Adwanikar H, Willis WD, Neugebauer V. Pain-related synaptic plasticity in spinal dorsal horn neurons: role of CGRP. *Mol Pain* 2006;2:31. [PubMed: 17002803]

8. Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* 2004;84:903–934. [PubMed: 15269340]
9. Brain SD, Williams TJ. Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. *Br J Pharmacol* 1985;86:855–860. [PubMed: 2416378]
10. Burstein R. Deconstructing migraine headache into peripheral and central sensitization. *Pain* 2001;89:107–110. [PubMed: 11166465]
11. Burstein R, Collins B, Jakubowski M. Defeating migraine pain with triptans: a race against the development of cutaneous allodynia. *Ann Neurol* 2004;55:19–26. [PubMed: 14705108]
12. Burstein R, Cutrer MF, Yarnitsky D. The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. *Brain* 2000;123 (Pt 8):1703–1709. [PubMed: 10908199]
13. Burstein R, Jakubowski M. Analgesic triptan action in an animal model of intracranial pain: a race against the development of central sensitization. *Ann Neurol* 2004;55:27–36. [PubMed: 14705109]
14. Burstein R, Levy D, Jakubowski M. Effects of sensitization of trigeminovascular neurons to triptan therapy during migraine. *Rev Neurol (Paris)* 2005;161:658–660. [PubMed: 16141951]
15. Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH. An association between migraine and cutaneous allodynia. *Ann Neurol* 2000;47:614–624. [PubMed: 10805332]
16. Cottrell GS, Roosterman D, Marvizon JC, Song B, Wick E, Pikios S, Wong H, Berthelie C, Tang Y, Sternini C, Bunnett NW, Grady EF. Localization of calcitonin receptor-like receptor and receptor activity modifying protein 1 in enteric neurons, dorsal root ganglia, and the spinal cord of the rat. *J Comp Neurol* 2005;490:239–255. [PubMed: 16082677]
17. Cridland RA, Henry JL. Effects of intrathecal administration of neuropeptides on a spinal nociceptive reflex in the rat: VIP, galanin, CGRP, TRH, somatostatin and angiotensin II. *Neuropeptides* 1988;11:23–32. [PubMed: 2452992]
18. Cumberbatch MJ, Williamson DJ, Mason GS, Hill RG, Hargreaves RJ. Dural vasodilation causes a sensitization of rat caudal trigeminal neurones in vivo that is blocked by a 5-HT1B/1D agonist. *Br J Pharmacol* 1999;126:1478–1486. [PubMed: 10217543]
19. Dubois NC, Hofmann D, Kaloulis K, Bishop JM, Trumpp A. Nestin-Cre transgenic mouse line Nestin-Cre1 mediates highly efficient Cre/loxP mediated recombination in the nervous system, kidney, and somite-derived tissues. *Genesis* 2006;44:355–360. [PubMed: 16847871]
20. Ebersberger A, Charbel Issa P, Vanegas H, Schaible HG. Differential effects of calcitonin gene-related peptide and calcitonin gene-related peptide 8-37 upon responses to N-methyl-D-aspartate or (R, S)-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate in spinal nociceptive neurons with knee joint input in the rat. *Neuroscience* 2000;99:171–178. [PubMed: 10924961]
21. Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS. Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. *Neuroscience* 1995;66:443–458. [PubMed: 7477885]
22. Galeazza MT, Stucky CL, Seybold VS. Changes in [125I]hCGRP binding in rat spinal cord in an experimental model of acute, peripheral inflammation. *Brain Res* 1992;591:198–208. [PubMed: 1332803]
23. Gallai V, Sarchielli P, Floridi A, Franceschini M, Codini M, Glioti G, Trequattrini A, Palumbo R. Vasoactive peptide levels in the plasma of young migraine patients with and without aura assessed both interictally and ictally. *Cephalalgia* 1995;15:384–390. [PubMed: 8536297]
24. Goadsby PJ. Migraine pathophysiology. *Headache* 2005;45 (Suppl 1):S14–24. [PubMed: 15833086]
25. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol* 1990;28:183–187. [PubMed: 1699472]
26. Hay DL, Poyner DR, Sexton PM. GPCR modulation by RAMPs. *Pharmacol Ther* 2006;109:173–197. [PubMed: 16111761]
27. Ho TW, Mannix LK, Fan X, Assaid C, Furtek C, Jones CJ, Lines CR, Rapoport AM. Randomized controlled trial of an oral CGRP receptor antagonist, MK-0974, in acute treatment of migraine. *Neurology* 2008;70:1304–1312. [PubMed: 17914062]
28. Juhasz G, Zsombok T, Modos EA, Olajos S, Jakab B, Nemeth J, Szolcsanyi J, Vitrai J, Bagdy G. NO-induced migraine attack: strong increase in plasma calcitonin gene-related peptide (CGRP)

- concentration and negative correlation with platelet serotonin release. *Pain* 2003;106:461–470. [PubMed: 14659530]
29. Kar S, Rees RG, Quirion R. Altered calcitonin gene-related peptide, substance P and enkephalin immunoreactivities and receptor binding sites in the dorsal spinal cord of the polyarthritic rat. *Eur J Neurosci* 1994;6:345–354. [PubMed: 7517279]
 30. Landy S, Rice K, Lobo B. Central sensitisation and cutaneous allodynia in migraine: implications for treatment. *CNS Drugs* 2004;18:337–342. [PubMed: 15089101]
 31. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. *Cephalalgia* 2002;22:54–61. [PubMed: 11993614]
 32. Li D, Ren Y, Xu X, Zou X, Fang L, Lin Q. Sensitization of Primary Afferent Nociceptors Induced by Intradermal Capsaicin Involves the Peripheral Release of Calcitonin Gene-Related Peptide Driven by Dorsal Root Reflexes. *J Pain*. 2008
 33. Lipton RB, Bigal ME, Ashina S, Burstein R, Silberstein S, Reed ML, Serrano D, Stewart WF. Cutaneous allodynia in the migraine population. *Ann Neurol* 2008;63:148–158. [PubMed: 18059010]
 34. LoPinto C, Young WB, Ashkenazi A. Comparison of dynamic (brush) and static (pressure) mechanical allodynia in migraine. *Cephalalgia* 2006;26:852–856. [PubMed: 16776701]
 35. Marvizon JC, Perez OA, Song B, Chen W, Bunnett NW, Grady EF, Todd AJ. Calcitonin receptor-like receptor and receptor activity modifying protein 1 in the rat dorsal horn: localization in glutamatergic presynaptic terminals containing opioids and adrenergic alpha2C receptors. *Neuroscience* 2007;148:250–265. [PubMed: 17614212]
 36. Mathew NT, Kailasam J, Seifert T. Clinical recognition of allodynia in migraine. *Neurology* 2004;63:848–852. [PubMed: 15365135]
 37. McCulloch J, Uddman R, Kingman TA, Edvinsson L. Calcitonin gene-related peptide: functional role in cerebrovascular regulation. *Proc Natl Acad Sci U S A* 1986;83:5731–5735. [PubMed: 3488550]
 38. Mogil JS, Miermeister F, Seifert F, Strasburg K, Zimmermann K, Reinold H, Austin JS, Bernardini N, Chesler EJ, Hofmann HA, Hordo C, Messlinger K, Nemmani KV, Rankin AL, Ritchie J, Siegling A, Smith SB, Sotocinal S, Vater A, Lehto SG, Klusmann S, Quirion R, Michaelis M, Devor M, Reeh PW. Variable sensitivity to noxious heat is mediated by differential expression of the CGRP gene. *Proc Natl Acad Sci U S A* 2005;102:12938–12943. [PubMed: 16118273]
 39. Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M. Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. *Pain* 1999;80:67–82. [PubMed: 10204719]
 40. Neugebauer V, Rumenapp P, Schaible HG. Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn-neurons during development of acute inflammation. *Neuroscience* 1996;71:1095–1109. [PubMed: 8684614]
 41. Olesen J, Diener HC, Husstedt IW, Goadsby PJ, Hall D, Meier U, Pollentier S, Lesko LM. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. *N Engl J Med* 2004;350:1104–1110. [PubMed: 15014183]
 42. Petersen KA, Birk S, Lassen LH, Kruuse C, Jonassen O, Lesko L, Olesen J. The CGRP-antagonist, BIBN4096BS does not affect cerebral or systemic haemodynamics in healthy volunteers. *Cephalalgia* 2005;25:139–147. [PubMed: 15658951]
 43. Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, Muff R, Fischer JA, Foord SM. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev* 2002;54:233–246. [PubMed: 12037140]
 44. Qi JS, Schulingkamp R, Parry TJ, Colburn R, Stone D, Haertlein B, Minor LK, Andrade-Gordon P, Damiano BP. Urotensin-II induces ear flushing in rats. *Br J Pharmacol* 2007;150:415–423. [PubMed: 17211454]
 45. Ruda MA, Ling QD, Hohmann AG, Peng YB, Tachibana T. Altered nociceptive neuronal circuits after neonatal peripheral inflammation. *Science* 2000;289:628–631. [PubMed: 10915627]
 46. Russo AF. Ramping it up: RAMP1 and the implications for migraine. *Pharmacogenomics* 2007;8:687–690. [PubMed: 18240900]

47. Schaible HG. On the role of tachykinins and calcitonin gene-related peptide in the spinal mechanisms of nociception and in the induction and maintenance of inflammation-evoked hyperexcitability in spinal cord neurons (with special reference to nociception in joints). *Prog Brain Res* 1996;113:423–441. [PubMed: 9009749]
48. Sun RQ, Lawand NB, Lin Q, Willis WD. Role of calcitonin gene-related peptide in the sensitization of dorsal horn neurons to mechanical stimulation after intradermal injection of capsaicin. *J Neurophysiol* 2004;92:320–326. [PubMed: 15212441]
49. Sun RQ, Lawand NB, Willis WD. The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. *Pain* 2003;104:201–208. [PubMed: 12855330]
50. Sun RQ, Tu YJ, Lawand NB, Yan JY, Lin Q, Willis WD. Calcitonin gene-related peptide receptor activation produces PKA- and PKC-dependent mechanical hyperalgesia and central sensitization. *J Neurophysiol* 2004;92:2859–2866. [PubMed: 15486424]
51. Tvedskov JF, Lipka K, Ashina M, Iversen HK, Schifter S, Olesen J. No increase of calcitonin gene-related peptide in jugular blood during migraine. *Ann Neurol* 2005;58:561–568. [PubMed: 16178016]
52. Tzabazis AZ, Pirc G, Votta-Velis E, Wilson SP, Laurito CE, Yeomans DC. Antihyperalgesic effect of a recombinant herpes virus encoding antisense for calcitonin gene-related peptide. *Anesthesiology* 2007;106:1196–1203. [PubMed: 17525595]
53. Williamson DJ, Hargreaves RJ. Neurogenic inflammation in the context of migraine. *Microsc Res Tech* 2001;53:167–178. [PubMed: 11301492]
54. Zhang Z, Dickerson IM, Russo AF. Calcitonin gene-related peptide receptor activation by receptor activity-modifying protein-1 gene transfer to vascular smooth muscle cells. *Endocrinology* 2006;147:1932–1940. [PubMed: 16373421]
55. Zhang Z, Winborn CS, Marquez de Prado B, Russo AF. Sensitization of calcitonin gene-related peptide receptors by receptor activity-modifying protein-1 in the trigeminal ganglion. *J Neurosci* 2007;27:2693–2703. [PubMed: 17344407]

Figure 1. RAMP1 mRNA levels in the spinal cord

(A) Schematic of the CGRP receptor. The calcitonin-like receptor (CLR), receptor activity modifying protein-1 (RAMP1), and receptor component protein (RCP) are shown in the plasma membrane. CGRP binding requires both CLR and RAMP1. (B) Levels of mouse (m), human (h), and total RAMP1 mRNA in the lumbar spinal cord were measured by Q-PCR. RNA was isolated and amplified in duplicate from nestin/hRAMP1 mice and nestin-cre control littermates. The copies of mouse and human RAMP1 were determined by a standard curve and normalized to GAPDH. The numbers of mice are indicated in parentheses and the mean $\pm$ SEM are given, asterisk indicates $p < 0.05$ between nestin/hRAMP1 and control.

Figure 2. Baseline mechanical responses are similar in nestin/hRAMP1 mice and littermate controls
 The response frequencies (percentage of responses to 5 filament applications averaged for both hindpaws) are shown for von Frey filaments E to J that have increasing forces of 1.5, 3, 5, 9.8, 13.7 and 19.6 mNewtons, as indicated. The numbers of mice are indicated in parentheses. Symbols are the mean $\pm$ SEM.

Figure 3. Intrathecal CGRP-evoked mechanical allodynia

All panels show the response frequency to 5 applications of the F filament following intrathecal injection of CGRP, CGRP(8-37), or PBS. (A) Injection of 1 or 5 nmol CGRP or PBS in control mice. (B) Injection of 1 nmol CGRP, PBS, or 1 nmol CGRP + 5 nmol CGRP(8-37) in nestin/hRAMP1 mice. Injection of 5 nmol CGRP(8-37) or PBS in nestin/hRAMP1 mice. PBS data from panels B is repeated in panels C for comparison purposes. The numbers of mice are indicated in parentheses. Symbols are the mean $\pm$ SEM. Asterisk indicates $p < 0.05$ between CGRP and PBS or CGRP(8-37) and PBS at the indicated time points.

Figure 4. Intraplantar CGRP does not increase mechanical nociception

All panels show the response frequency to 5 applications of the F filament following intraplantar injection of 1.3 nmol CGRP or PBS. (A) Responses in the ipsilateral paw in control and (B) nestin/hRAMP1 mice. (C) Responses in the contralateral paw in control and (D) nestin/hRAMP1 mice. The numbers of mice are indicated in parentheses. Symbols are the mean $\pm$ SEM.

Figure 5. Sensitized response of the nestin/hRAMP1 mice to intraplantar capsaicin

All panels show the response frequency to 5 applications of the F filament following intraplantar injection of capsaicin. (A) Responses in the ipsilateral paw in control and nestin/hRAMP1 mice following injection of 0.01% capsaicin. (B) Responses in the contralateral paw in control and nestin/hRAMP1 mice following injection of 0.01% capsaicin. (C) Responses in the ipsilateral paw in control and nestin/hRAMP1 mice following injection of 0.001% capsaicin. (D) Responses in the contralateral paw in control and nestin/hRAMP1 mice following injection of 0.001% capsaicin. The numbers of mice are indicated in parentheses. Symbols are the mean $\pm$ SEM. Asterisk indicates $p < 0.05$ between nestin/hRAMP1 and control at the indicated time points.