B-type natriuretic peptide is neither itch-specific nor functions upstream of the GRP-GRPR signaling pathway

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B-type natriuretic peptide is neither itch-specific nor functions upstream of the GRP-GRPR signaling pathway

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Abstract

Background: A recent study by Mishra and Hoon identified B-type natriuretic peptide (BNP) as an important peptide for itch transmission and proposed that BNP activates spinal natriuretic peptide receptor-A (NPRA) expressing neurons, which release gastrin releasing peptide (GRP) to activate GRP receptor (GRPR) expressing neurons to relay itch information from the periphery to the brain (Science 340:968–971, 2013). A central premise for the validity of this novel pathway is the absence of GRP in the dorsal root ganglion (DRG) neurons. To this end, they showed that Grp mRNA in DRG neurons is either absent or barely detectable and claimed that BNP but not GRP is a major neurotransmitter for itch in pruriceptors. They showed that NPRA immunostaining is perfectly co-localized with Grp-eGFP in the spinal cord, and a few acute pain behaviors in Nppb−/− mice were tested. They claimed that BNP is an itch-selective peptide that acts as the first station of a dedicated neuronal pathway comprising a GRP-GRPR cascade for itch. However, our studies, along with the others, do not support their claims.

Findings: We were unable to reproduce the immunostaining of BNP and NPRA as shown by Mishra and Hoon. By contrast, we were able to detect Grp mRNA in DRGs using in situ hybridization and real time RT-PCR. We show that the expression pattern of Grp mRNA is comparable to that of GRP protein in DRGs. Pharmacological and genetic blockade of GRP-GRPR signaling does not significantly affect intrathecal BNP-induced scratching behavior. We show that BNP inhibits inflammatory pain and morphine analgesia.

Conclusions: Accumulating evidence demonstrates that GRP is a key neurotransmitter in pruriceptors for mediating histamine-independent itch. BNP-NPRA signaling is involved in both itch and pain and does not function upstream of the GRP-GRPR dedicated neuronal pathway. The site of BNP action in itch and pain and its relationship with GRP remain to be clarified.

Keywords: BNP, NPRA, GRP, GRPR, Itch, Pain, Spinal cord, DRG

Background

Identification of novel itch mediators in sensory neurons and in the spinal cord and elucidation of the molecular and cellular circuitry have become a new and exciting frontier for the past several years. As many itch mediators (neuropeptides, receptors and channels etc.) have been implicated in itch transmission, conflicting results and confusion emerge. Gastrin releasing peptide (GRP) is a neuropeptide expressed in primary afferents and is necessary for relaying non-histaminergic itch from the skin to the spinal cord [1-4]. For several decades, the availability of the antibody against the highly conserved nine amino acids in the C terminus of GRP/bombesin across species has enabled the demonstration of expression of GRP in primary afferents of rat, mouse, cat, monkey and human. Using the anti-GRP or bombesin antibody, the percentage of GRP+ cells has been consistently estimated to be around 5–8% in dorsal root ganglion (DRG) neurons and 12% in trigeminal ganglia (TG) neurons of rodents [2,5-14]. Several groups have used dorsal root rhizotomy to examine the origin of GRP+.
primary afferents. For the past 30 years, all the studies have consistently found that the majority of GRP+ fibers are derived from primary afferents [2,4,7-9,15], with only one exception [16]. The earlier findings on the origin of GRP+ fibers have been reviewed previously [1,17]. In addition, neonatal capsaicin treatment dramatically depleted GRP+ fibers in the spinal cord, which supported the notion that the source of GRP is from primary afferents [18]. It is important to note that the specificity of the anti-Grp antibody has been repeatedly confirmed using Grp-/+ mice [4,19] and by an antigen absorption approach [16], the latter of which was first performed more than 30 years ago [8]. Importantly, several studies found that GRP expression was markedly enhanced in primary afferents in mice, monkeys and patients with various chronic itch conditions [4,11-13], matching an up-regulation of GRPR in the spinal cord [4,12]. Those studies support a physiological role of GRP in primary afferents. On the other hand, the specific expression of Grp mRNA in lamina I of the spinal cord and a failure to detect Grp mRNA in DRG neurons using in situ hybridization (ISH) by some investigators have reignited interests in spinal Grp expression [16]. To date, little evidence exists to support that Grp mRNA in lamina I of the spinal cord is in fact translated into GRP protein because none of the GRP immunohistochemistry studies after dorsal root rhizotomy have revealed the neuronal cell body staining pattern that resembles that of Grp mRNA in the spinal cord. It was speculated that some of the residual GRP+ fiber staining in the spinal cord after rhizotomy could be of descending origins [8,9]. Moreover, an up-regulation of GRP protein intrinsic to the dorsal horn in the setting of chronic itch was not detected [4], further arguing against the physiological role of Grp mRNA in the spinal cord. Additional evidence supporting the idea of activation of postsynaptic GRPR by presynaptic GRP release has also emerged. Potential synaptic contacts between Mrgrpa3+ primary afferents and GRPR+ neurons were demonstrated [20]. Electron microscopic analysis revealed that GRP+ terminals contained large dense-core vesicles that formed synaptic contacts with a few dendrites of dorsal horn neurons [15]. Despite a large body of literature that demonstrated the presence of GRP protein in DRGs and primary afferents, a recent study by Mishra and Hoon attempted to show a lack of Grp mRNA in sensory neurons and proposed that B-type natriuretic peptide (BNP), rather than GRP, is a major neuropeptide in primary afferents for itch transmission [21]. Further, they proposed that BNP activates natriuretic peptide receptor-A (NPRA) expressing neurons in the spinal cord, which in turn release GRP to activate GRPR+ neurons. In this report, we present the data that do not support their conclusions.

**Grp mRNA expression in DRG neurons**

A central premise for the validity of the BNP-NPRA-Grp-GRPR cascade proposed by Mishra and Hoon is a lack of GRP in primary afferents. Although a specific GRP antibody has eliminated the necessity for examining Grp mRNA using ISH technique, single cell RT-PCR permits a more precise assessment of co-expression of Grp mRNA with other markers such as Trpv1 mRNA in a subset of DRG neurons [28,29]. Instead of using anti-GRP antibody
or single cell RT-PCR, Mishra and Hoon presented three pieces of evidence to dispute the presence of Grp mRNA in DRGs [21]. First, they could not detect Grp mRNA in DRGs using ISH. Unfortunately, Grp mRNA detection in DRGs by ISH has remained a challenge for some investigators [16,21]. We have recently shown that BRAFNav1.8 mice represent a unique animal model for investigating the mechanisms of chronic itch [4]. In BRAFNav1.8 mice, the expression of a cohort of itch-related mediators including GRP protein was markedly increased in DRGs, which account for the development of spontaneous itch in these mice [4]. Using ISH, we detected a few Grp+ cells in wild-type (WT) DRGs, consistent with GRP immunostaining pattern (Figure 2A). In contrast, many more Grp+ cells were detected in DRGs of BRAFNav1.8 mice, demonstrating enhanced Grp mRNA expression in the setting of chronic itch (Figure 2A), consistent with enhanced GRP protein expression as detected using anti-GRP antibody [4]. These conflicting results indicate that the sensitivity of ISH rests on multiple factors (e.g., tissues, age of animals, perfusion method, the abundance of transcripts, etc.). Concerning gene expression in DRGs and spinal cord, it is not uncommon that mRNA or protein for some genes can be easily detected in one tissue but not the other. Therefore, ISH procedure or immunostaining procedure has to be optimized and tailored to specific cRNA probes/antibodies if the routine procedure fails to work.

Second, Mishra and Hoon tried to minimize the presence of Grp mRNA by stating that they were unable to detect more than “trace quantities” of Grp expression in DRGs using a “sensitive quantitative” PCR assay [21]. However, “trace quantities” of Grp could be a sufficient amount of mRNA to generate physiologically relevant level of GRP that is necessary for GRPR activation in the spinal cord, particularly given that GRP can bind and
activate GRPR at nanomolar concentrations [30]. Using real-time RT-PCR and validated Grp primers, we detected a dramatic up-regulation of Grp mRNA in BRAFNav1.8 DRGs (Figure 2B), in line with the ISH results (Figure 2A). Grp cDNA was amplified from as low as 0.01 μl of WT DRG cDNA while no amplification was seen when using up to 1 μl of control samples (ΔRT) in which reverse transcriptase was omitted (Figure 2C). The single narrow peak at 83°C in melt curve further validated the high quality of the reactions (Figure 2D).

Finally, Mishra and Hoon used Grp-eGFP transgenic mice generated by GENSAT as evidence to argue for an absence of Grp mRNA expression in DRGs. It has been well known that transgenic eGFP mouse lines generated by BAC engineering-based technique may not necessarily recapitulate endogenous expression as over-expression or an absence of eGFP expression are two frequent problems encountered, which can be attributable to the integration site and copy number of the transgene in the mouse genome and the size of the genomic DNAs used to construct the vector. Although BAC-based transgenic lines serve as a great tool for researchers to follow endogenous expression of many genes, it is important to validate the line using either functional or molecular approaches. A common practice is to screen the transgenic mice derived from pronuclear injection of a BAC vector and to identify the founder that may best mimic the endogenous gene expression, since there are great variations of transgene expression among the transgenic mice. One can find more information at GENSAT website: http://www.gensat.org/index.html about the discrepancies between eGFP expression patterns generated by GENSAT and those in literature. It was also not rare to see discrepancies in the eGFP expression patterns for the same gene between different mouse lines when different BAC vectors were used. For example, two independent lines of Grp-cre mice (KH-107 and KH-288) generated by GENSAT exhibit distinct and overlapping expression patterns.
patterns in some areas of the brain. Therefore, the expression pattern of a transgenic line cannot be considered as valid evidence to argue for an absence of the expression of a gene.

**BNP functions independent of GRP and GRPR**

If BNP-NPRA signaling depends on the activation of GRPR by GRP, intrathecal (i.t.) BNP-elicited scratching behavior would be lost or attenuated in **Grpr** knock-out (KO) or **Grp** KO mice. However, we did not observe significant difference in BNP-elicited scratching responses between **Grpr** KO mice and their WT littermates (Figure 3A, 3B). Next we examined whether i.t. BNP-induced scratching behavior could be attenuated by 77427 (Chembridge), a GRP blocker whose specificity was recently confirmed [4]. 77427 effectively reduced i.t. GRP-induced scratching behavior (Figure 3C). In contrast, i.t. BNP-induced scratching behavior was not inhibited by coinjection of 77427 (Figure 3D). Similarly, **Grp** KO mice and their WT littermates showed comparable scratching behavior evoked by i.t. BNP (Figure 3E). These genetic and pharmacological blockade studies demonstrate that GRP-GRPR signaling does not act downstream of BNP in relaying itch information. Notably, the time-course of scratching responses evoked by i.t. BNP and GRP differs significantly. In WT mice, i.t. BNP (1 nmol) induced mild bilateral scratching behavior in the first 30 min and the scratching behavior was gradually increased in the second 30 min during the one-hour observation (Figure 3F, 3G). In contrast, i.t. GRP (1 nmol) induced robust scratching responses for about 10 min after injection and the number of scratching bouts decreased gradually within 40 min. Nonetheless, these interesting and apparent differences in pharmacokinetics of the two peptides...
further argue against the possibility that BNP and GRP function in the same pathway.

**Intrathecal BNP inhibits inflammatory pain and morphine analgesia**

Mishra and Hoon tested a few acute pain behaviors in Nppb−/− mice and proposed that BNP is an “itch-selective” neuropeptide that acts as the first station in primary afferents to activate the GRP-GRPR dedicated neuronal pathway for itch via spinal NPRA [21]. However, BNP and NPRA in DRG neurons have been suggested to be a novel auto-feedback nociceptive pathway to modulate inflammatory pain [26] and BNP is a negative regulator of neuronal excitability in response to nociceptive stimuli [27]. Moreover, i.t. BNP attenuated morphine analgesia in mice [31].

We next examined the role of BNP in nociceptive processing. Although BNP (0.04 nmol) did not alter acute thermal pain as tested in 50°C tail-immersion assay (Figure 4A), it significantly reduced the licking and flinching time induced by intraplantar (i.pl.) injection of capsaicin (Figure 4B). i.t. BNP also markedly attenuated formalin-evoked nocifensive behavior during phase II response (Figure 4C) and reduced morphine analgesia (Figure 4D). These results confirm that BNP plays an important role in nociceptive processing in mice. In contrast, neither GRP nor GRPR is involved in morphine analgesia [32] and both loss- and gain-of-function studies consistently demonstrated that GRP-GRPR signaling is not involved in nociceptive processing [2-4,32]. Therefore, the BNP-NPRA and GRP-GRPR pathways are unlikely to be in the same dedicated neuronal pathway for itch.

**Conclusions**

The present study questions the experimental approaches that Mishra and Hoon used and the data they presented in their studies. Specifically, we question the BNP immuno-staining pattern in DRGs as we could not recapitulate their results in DRG neurons using the same antibody. For NPRA staining, we detected numerous NPRA+ neurons in DRGs and NPRA+ fibers in the spinal cord and were unable to detect lamina I-restricted NPRA+ neuronal staining pattern that resembles that of Grp mRNA expression in the spinal cord. We show that BNP-NPRA signaling is involved in both itch and pain, which contrasts with itch-specific GRP-GRPR signaling. Given that BNP could activate peripheral NPRA to reduce the excitability of sensory neurons and the conflicting data on BNP expression in sensory neurons [26,27], we suggest that the sites of action (glia vs neurons; DRGs vs spinal cord) and the underlying mechanisms (inhibitory vs excitatory) for BNP and NPRA in itch and pain remain to be clarified.

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**Figure 4 BNP inhibits inflammatory pain.** A) BNP (0.04 nmol, i.t.) did not alter the baseline of thermal sensation as tested in a 50°C tail-immersion assay. n = 9, p > 0.05, one-way ANOVA. B) BNP significantly reduced the licking and flinching time induced by capsaicin (2 μg, i.pl.) injection. n = 10. *p < 0.05 versus saline, unpaired t test. C) BNP (0.1 nmol, i.t.) did not change the first phase (0–10 min), but significantly reduced the second phase (10–60 min) of formalin pain. n = 6–7. *p < 0.05 versus saline, unpaired t test. D) Pre-injection of BNP (0.04 nmol, i.t.) 10 min before morphine injection (5 mg/kg, i.p.) significantly blocked morphine analgesic effect as tested by tail-immersion assay 60 min after morphine injection. n = 6. *p < 0.05 versus pre-saline + morphine, unpaired t test.
We demonstrate that Grp mRNA is detectable by ISH and by real-time RT-PCR in DRGs. More important, we demonstrate that BNP-NPRA signaling does not function as the first station upstream of the GRP-GRPR pathway using both pharmacological and genetic approaches, the latter of which is considered to be superior to the former. Taken together, abundant evidence supports the notion that GRP is indeed a primary afferent transmitter for itch transmission provides an important and new avenue for further investigating itch signaling from the prurceptors to the spinal cord. The action of BNP-NPRA in itch and pain and their relationship with GRP-GRPR signaling require more extensive investigation.

Materials and methods

Animals

Male mice between 7 and 12 weeks old were used for the experiments. C57BL/6 J mice were purchased from the Jackson Laboratory. Grp KO mice, Grpr KO mice and BRAFNav1.8 mice were used as described [2,4]. All the mice were housed under a 12 h light/dark cycle with food and water provided ad libitum. All the 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 ISH

Immunohistochemical staining was performed as described [33]. Briefly, mice were anesthetized with an overdose of ketamine/xylazine cocktail and fixed by intracardiac perfusion of cold 0.01 M PBS (pH 7.4) and 4% paraformaldehyde. Tissues were immediately removed, post-fixed in the same fixative overnight at 4°C, and cryoprotected in 30% sucrose PBS solution. Tissues were frozen and sectioned at 20 μm thickness on a cryostat. Free-floating sections were blocked in a solution containing 2% donkey serum and 0.3% Triton X-100 in PBS for 1 h at room temperature. The sections were incubated with primary antibodies overnight at 4°C followed by secondary antibodies. The following primary antibodies were used at the specified dilutions: rabbit anti-NPRA (1:200, LS-C109432, LifeSpan Bioscience), rabbit anti-NPRA (1:300, ab70848, Abcam), mouse anti-BNP (1:100, LS-C82084, LifeSpan Bioscience), mouse anti-NeuN (1:10,000, MAB377, Millipore), and goat anti-BNP (V-17) (1:100, sc-67455, Santa Cruz). The secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. including Cy3- or FITC-conjugated donkey anti-rabbit or anti-mouse IgG (1:1,000). For the ISH study, a digoxigenin-labeled cRNA probe was used as described earlier [34]. Images were taken using a Nikon Eclipse Ti-U microscope and confocal microscope (Leica TCS SPE).

Real-time RT-PCR

Real-time RT-PCR was performed as previously described [32]. Briefly, DRGs were dissected out from 9 weeks old male mice (n = 4 per genotype). Tissues were temporarily stored on ice in 1 ml of RNA stabilizer (RNAlater, Qiagen). Total RNA was isolated and genomic DNA was removed in accordance with manufacturer’s instructions (RNeasy mini kit; Qiagen). RNA was quantified using Eppendorf BioPhotometer and stored at −80°C. Single-stranded cDNA was synthesized from 1 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies) and stored at −20°C until the analysis. Reverse transcriptase was omitted for negative control (ΔRT). Gene expression of Grp was determined by real-time PCR (StepOnePlus; Applied Biosystems). Specific primers spanning intron were designed with the NCBI Primer-BLAST. The fidelity and specificity of the primers were validated by real-time PCR using serial volume (1 μl, 0.1 μl and 0.01 μl) of WT DRG cDNA and PCR efficiency (Ef) was calculated. The primers used are:

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Grp</td>
<td>5′-GGTGCCGTGAGAGAAT-3′ (exon1)</td>
<td>5′-GGGTTCAATTAAT-3′ (exon3)</td>
</tr>
<tr>
<td>Actb</td>
<td>5′-TGTTACCAACTGGGACGACA-3′ (exon3)</td>
<td>5′-GGGGTGTTGAAGGTCCAGCAA-3′ (exon4)</td>
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Total RNA (1 μg) was used for cDNA synthesis. cDNA (1 μl) was assayed in duplicates. Thermal cycling was initiated with denaturation at 95°C for 10 min. After this initial step, 40 cycles of PCR (heating at 95°C for 10 sec and 60°C for 30 sec) were performed. Data were analyzed using Comparative CT Method (StepOne Software v2.2.2) and the expression of Grp was normalized to the expression of Actb after adjustment with Ef.

Behavioral tests

Behavioral tests were videotaped (HDR-CX190, Sony) from a side angle. The videos were played back on a computer and the quantification of mice behaviors were done by persons who were blinded to the treatments and genotypes.

Scratching behavior

Itch behaviors were performed as previously described [2,3]. Briefly, prior to experiments, mice were given 30 min to acclimate to the plastic arenas (10 × 10.5 × 15 cm). Mice were then briefly removed from the chamber for drug injections.
Thermal sensitivity
Thermal sensitivity was determined using tail immersion assay. Mice tails were dipped beneath the warm water (50°C) in a temperature-controlled water bath (IITC Inc.). The latency to withdrawal was measured with a 12-sec cutoff. For morphine analgesia study, BNP (Phoenix Pharmaceuticals) was intrathecally injected 10 min before morphine injection (5 mg/kg, i.p.) and tail immersion (50°C) results were expressed as percentage of maximum possible effect [%MPE = (post drug latency - pre drug latency) × 100/ (cutoff time – pre drug latency)].

Acute nociceptive behavior
Algogens were intraplantarily injected into right hindpaws. The duration of licking and flinching of the injected paw was recorded for 60 min after injection of formalin (2%, 20 μl) and for 5 min after injection of capsaicin (2 μg, 20 μl).

Data analysis
All values are expressed as the means ± standard error of the mean (S.E.M.). Statistical analysis was performed using Prism v5.03 (GraphPad Software). p < 0.05 was considered statistically significant.

Abbreviations
BNP: B-type natriuretic peptide; NPRA: Natriuretic peptide receptor-A; GRP: Gastrin releasing peptide; GRPR: Gastrin releasing peptide receptor; ISH: In situ hybridization; DRG: Dorsal root ganglia; TG: Trigeminal ganglia; i.t.: intrathecal.

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

Authors’ contributions
XYL, FQH, DB, HL performed immunostaining and ISH, ZFC and XYL wrote the manuscript. All authors supervised the project and ZFC and XYL read and approved the final manuscript.

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