ORIGINAL RESEARCH

Gastrin-Releasing Peptide/Gastrin-Releasing Peptide Receptor Participation in Itch Sensation Signaling in the Spinal Cord of Uremic Pruritus Mice

Huili Li, BD; Weiwei Gao, BD; Ze Zhang, MM; Hao Chen, BD; Yitong Wang, BD; Liang Du, BD

ABSTRACT

Background • Uremic pruritus is a prevalent clinical symptom in maintenance dialysis patients. Existing evidence establishes a connection between itch transmission and the gastrin-releasing peptide/gastrin-releasing peptide receptor signaling pathway.

Objective • To investigate the involvement of the gastrin-releasing peptide/gastrin-releasing peptide receptor in itch sensation signaling within the spinal cord of uremic pruritus.

Design • An animal study was conducted.

Setting • The research was conducted at the First Hospital of Hebei Medical University.

Participants • A total of 50 male C57BL/6J mice (weight: 30-40 g) were acquired from Beijing Weitonglihua Laboratory Animal Center.

Interventions • Mice were categorized into five groups: normal, sham, Y, A, and B. The Y group received intrathecal injections of saline (5 ul). The A group received intrathecal injections of gastrin-releasing peptide (0.1 nmol, 5 ul), and the B group received intrathecal injections of the gastrin-releasing peptide receptor antagonist RC-3095 (0.3 mmol, 5 ul).

Primary Outcome Measures • (1) Pruritus behavior of mice and (2) expression of gastrin-releasing peptide, gastrin-releasing peptide receptor, and inositol trisphosphate.

Results • Scratching times in the Y group significantly surpassed those of normal and sham groups, increasing over time. Gastrin-releasing peptide and receptor expression rose in the uremic pruritus mouse model compared to normal and sham groups ($P < .05$). Expression of gastrin-releasing peptide and its receptor was significantly elevated in the uremic pruritus mouse model compared to the normal and sham groups ($P < .05$). Inositol trisphosphate expression in the dorsal spinal horn of Y group mice increased compared to normal and sham groups. Intrathecal gastrin-releasing peptide heightened inositol trisphosphate expression, while the peptide receptor antagonist RC-3095 reduced it. Y group scratching times were higher than normal and sham groups, increasing after intrathecal gastrin-releasing peptide but decreasing after RC-3095 injection.

Conclusion • The gastrin-releasing peptide/gastrin-releasing peptide receptor signaling pathway is involved in the development of uremic pruritus. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Uremic pruritus (UP) is a prevalent clinical symptom among maintenance dialysis patients.[1] According to three epidemiological reports from the dialysis outcomes and practice patterns study (DOPPS), 44.9%, 42%, and 44% of dialysis patients experienced pruritus.[2] Moreover, a thorough analysis, combining DOPPS with four additional epidemiological studies, revealed that 24.5% of dialysis patients presented with severe pruritus.[3]

Pruritus is notably linked to sleep disorders, depression, diminished quality of life, and heightened mortality in uremia patients, serving as a crucial indicator of poor clinical prognosis.[4] Despite its significant impact, the unclear pathogenesis of UP contributes to a lack of specific clinical treatments.[5] Thus, understanding the precise mechanism of UP and identifying effective treatment targets are imperative for enhancing clinical interventions.

The development of UP involves a complex pathophysiological process, linked with factors and mechanisms such as reduced skin moisture content, the immune inflammation hypothesis, the opioid hypothesis, accumulation of metabolic substances, and the histamine and parathyroid hormone mechanism.[6] Among these,

clinicians widely accept the accumulation of metabolic substances and the disturbance of calcium and phosphorus ion theory as significant contributors to UP.[7]

However, the above-mentioned factors have not definitively established a causal relationship with UP, and current treatments, including dialysis, remain suboptimal in alleviating the severity and reducing the frequency of itching.[8] Upon reviewing the literature, our research group observed that prior studies on UP primarily focused on mechanisms related to uremia, aiming to trace the origins and eliminate pruritus entirely.[9] However, we posit that the pruritic mediator itself may play a crucial role in UP.

The regulation of somatosensory transduction in the spinal cord is governed by monoaminergics.[10] As a vital aspect of an animal's adaptive response to changing environments, the promotion and inhibition of somatosensory transduction in the spinal cord are crucial for maintaining homeostasis.[11] Monoaminergic regulation of neural circuits plays a pivotal role in modulating behavioral responses to somatosensory inputs.[12] Numerous studies have indicated that the suppression of itch is correlated with activity levels in midbrain regions.[13]

Among the pruritic mediators identified to date, gastrin-releasing peptide (GRP) stands out as the central molecule mediating neurogenic pruritus.[14] Indeed, as implied by its name, GRP possesses the ability to actively stimulate the secretion of gastrin.[15] Human GRP, comprising 148 amino acids, is expressed in the gastrointestinal tract, dorsal root ganglion, and trigeminal ganglion cells.[16] The GRP secreted by dorsal root ganglion cells can be conveyed to the dorsal horn of the spinal cord through axons[17]

GRP binds to the GRPR, a member of the mammalian RANKL (Receptor Activator of Nuclear Factor **κB** Ligand) family, expressed in the spinal dorsal horn layer I neurons.[18] Notably, GRP/GRPR signaling has been implicated in colorectal cancer progression[19] and chemical activity associated with periodontal inflammation.[20] Crucially, past research has established a connection between GRP/GRPR signaling and itch transmission.[21] However, its involvement in the signaling of itchy sensation in the spinal cord of UP remains unclear.

In our study, we established a mouse model of chronic renal failure to examine the potential involvement of the GRP-GRPR signaling pathway in the development of UP. The findings from our study provide a valuable insight about the GRP-GRPR signaling pathway crucial role in the occurrence of UP, offering a novel perspective for the treatment of this condition.

MATERIALS AND METHODS

Experimental Animal Procurement and Care

A total of 50 male C57BL/6J mice weighing 30-40 g were sourced from Beijing Weitonglihua Laboratory Animal Center. The mice were provided with unrestricted access to food and water, maintained in a room with a light-dark cycle of 12 hours each at a temperature of 22-25°C, and a relative humidity range of 40%-60%.

Establishment of Chronic Renal Failure Mouse Model

The method for creating a chronic renal failure mouse model was implemented according to previously reported procedures.[22]

Pre-Modeling Preparations. One day before modeling, mice underwent fasting, bowel evacuation, and abdominal injection of 1% sodium pentobarbital for anesthesia.

Renal Electrocoagulation and Nephrectomy. In a concise summary of the procedure, electrocoagulation was applied to the entire surface of the right kidney, leaving a 2 mm intact tissue around the hilum unaffected. Subsequently, left nephrectomy was carried out 15 days later.

Sham Operation. In sham-operated mice, the right kidney underwent electrocoagulation, and the left kidney was temporarily exposed, following a procedure akin to nephrectomy. However, it is crucial to note that the actual nephrectomy procedure was not executed in this sham operation.

Anesthesia Approach. Employing detailed care, all surgeries were executed under anesthesia through small incisions, prioritizing the preservation of intestinal and upper abdominal integrity. This approach aimed to minimize interference and ensure precise surgical interventions.

Surgical Procedures for Renal Intervention. All animals underwent one of three procedures: (1) renal surface electrocoagulation; (2) nephrectomy; or (3) sham surgery. Renal electrocoagulation was executed using a foot-operated single-point cauterizer angled at 30°. Post-electrocoagulation, the kidney was carefully restored to the renal fossa and enveloped entirely by the tissue of the abdominal wall and skin.

Group Allocation and Intrathecal Injections

The mice were categorized into five groups (n=10): (1) normal group; (2) sham group; (3) Y group; (4) A group, and (5) B group. In the Y group, mice received intrathecal injections of saline (5 ul). The A group underwent intrathecal injections of GRP (0.1 nmol, 5 ul, Sigma, USA), while the B group received intrathecal injections of the GRPR antagonist RC-3095 (0.3 mmol, 5 ul, Sigma, USA).

Drug Administration and Pruritus Observation

After the successful modeling of UP, the drugs were administered twice daily for three consecutive days. Pruritus behavior changes were carefully observed one hour post-drug administration each day, extending until the fifth day after the drug was administered. It ensures sustained therapeutic impact, allowing for a comprehensive assessment of its effects on pruritus behavior. This approach offered a detailed understanding of the drug's prolonged efficacy.

Observation Indicators

Behavioral Assays: Observing Scratching Behavior. In the dedicated environment of a behavioral observation device, mice were carefully placed, and their activity was recorded for half an hour using a camera post-modeling. The criterion for a scratching event was defined as any mouse displaying scratching behavior within a 1-second timeframe.

Utilizing a controlled environment ensures accurate and consistent behavioral observations, eliminating external factors that may influence scratching behavior.

Enzyme-Linked Immunosorbent Assay (ELISA). The quantification of GRP and its receptor (GRPR) in blood samples was achieved through the utilization of a mice GRP (Cusabio, Wuhan, China) Kit and GRPR ELISA kit (Cusabio, Wuhan, China). ELISA provides a highly sensitive and specific method for the precise quantification of biomolecules, ensuring accurate measurement of GRP and GRPR levels in blood samples.

Immunofluorescence Staining. An immunofluorescence staining assay was employed to investigate the protein expression and subcellular localization of GRP and its receptor (GRPR). Mice tissues were first fixed using 3.7% formaldehyde, followed by permeabilization with 0.5% Triton X-100 at room temperature. Subsequent steps included two washes with phosphate-buffered saline (PBS) and blocking with 5% bovine serum albumin (BSA) for 1 hour at 37°C.

Cells were then incubated with primary antibodies overnight at 4°C. Post-PBS washing, cells received an Alexa Fluor® 647-labeled secondary antibody (1:200 dilution), with nuclei co-stained using 4',6-diamidino-2-phenylindole (DAPI). The resulting staining patterns were captured using an E100 optical microscope. Immunofluorescence staining allows for the visualization and localization of specific proteins within cells, providing insights into their distribution and interactions.

Western Blot Analysis

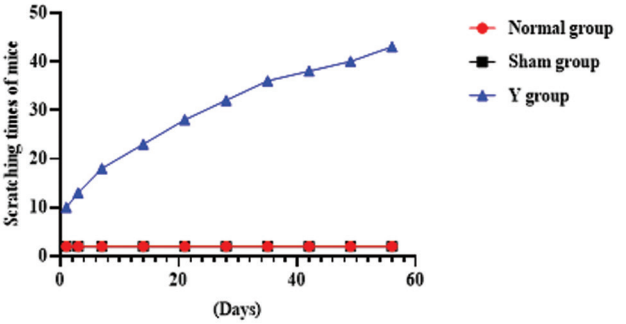
Mice spinal cord tissues, frozen with liquid nitrogen, were carefully ground, and protein extraction was performed using Lysis Buffer (Beyotime, China). After quantification of total protein concentrations using a Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, USA), the samples underwent separation on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, succeeded by electrophoretic transfer to polyvinylidene difluoride membranes.

Post-incubation with 5% non-fat milk for 1 hour, the membranes were probed with anti-GRP (1/500, GTX54161, GeneTex, USA), anti-GRPR (1/500, ab228759, Abcam, UK), or anti-β-actin (1/1000, ab8226, Abcam, UK) antibodies. Subsequently, the membranes were washed and treated with horseradish peroxidase-conjugated secondary antibodies (1/2000, ab6728, Abcam, UK) for 1 hour. Finally, the membranes were exposed to an electrochemiluminescence (ECL) solution (Millipore, Germany). Western blot analysis is an effective technique for evaluating protein expression levels and confirming the specificity of antibodies. This method ensures accurate detection and quantification of GRP, GRPR, and β-actin proteins, facilitating a comprehensive understanding of their presence and potential interactions in the spinal cord tissues of mice.

Statistical Analysis

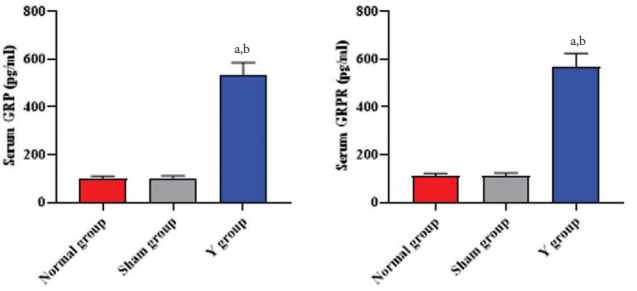
Statistical analyses were conducted using SPSS 19.0 software (IBM SPSS, USA). The significance of differences among groups was evaluated through one-way analysis of variance (ANOVA). Results were expressed as mean ± standard deviation ($\bar{x} \pm s$), with a $P < .05$ considered statistically significant.

Figure 1. Scratching Behavior Analysis



Note: The graph illustrates the scratching behavior of mice in different groups over time. The data depicts the frequency of scratching bouts, providing insights into pruritus severity and temporal patterns among the experimental groups.

Figure 2. Serum Levels of GRP and GRPR in UP Mice Model



#$P < .05$ when compared to the normal group
*$P < .05$ when compared to the sham group.

Note: ELISA analysis revealed the elevated serum levels of gastrin-releasing peptide (GRP) and its receptor (GRPR) in the experimental groups.

Abbreviations: GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor.

RESULTS

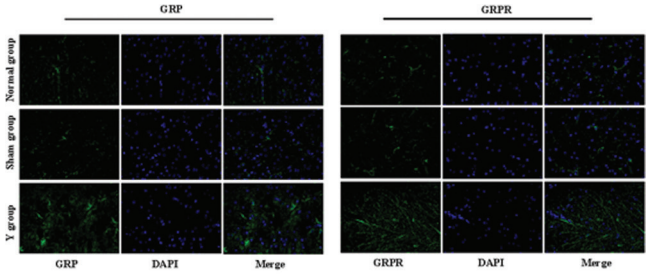
Observation of Mice Pruritus Behavior in Each Group

Scratching times in the Y group exhibited a progressive increase over time, demonstrating a noteworthy elevation compared to both normal and sham groups, see Figure 1. Continuous monitoring of scratching behavior provides a dynamic understanding of pruritus progression and treatment efficacy.

Expression of GRP and GRPR in Uremic Pruritus (UP)

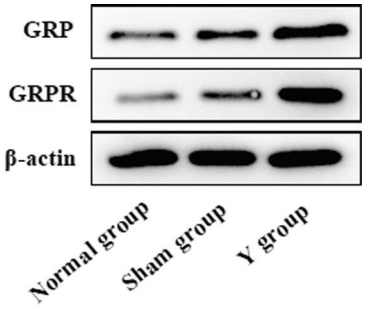
Figure 2 illustrates elevated serum levels of GRP and its receptor (GRPR) in the UP mice model, compared to both the normal and sham groups ($P < .05$). Additionally, immunofluorescence staining and western blot analysis, refer to Figure 3 and Figure 4, further confirm heightened expression of GRP and GRPR in the dorsal spinal horn of mice in the Y group when compared to the normal and sham groups.

Figure 3. GRP and GRPR Expression in Dorsal Spinal Horn



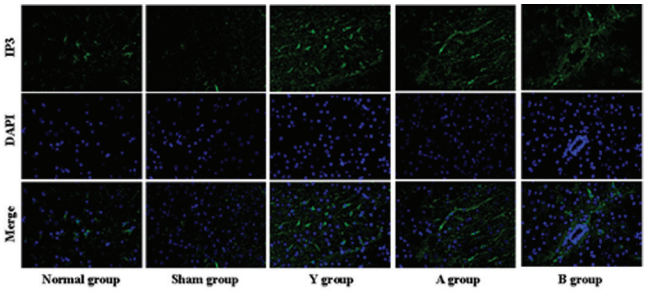
Note: Immunofluorescence staining displayed the expression levels of gastrin-releasing peptide (GRP) and its receptor (GRPR) in the dorsal spinal horn of mice across various groups. DAPI (4',6-diamidino-2-phenylindole) was used for nuclei co-staining.

Figure 4. Western Blot Analysis of GRP and GRPR Expression in Dorsal Spinal Horn



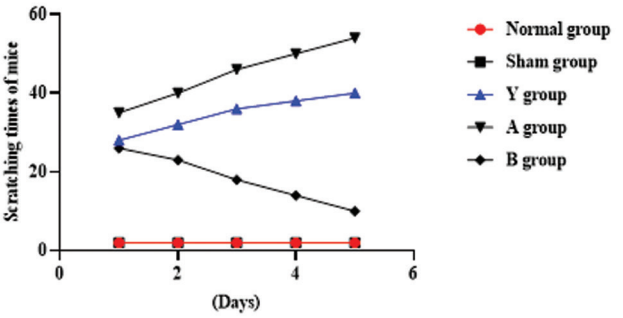
Note: Western blot analysis was conducted to assess the protein levels of gastrin-releasing peptide (GRP) and its receptor (GRPR) expression in the dorsal spinal horn of mice across different groups.

Figure 5. Immunofluorescence Staining of IP3 Expression in Dorsal Spinal Horn



Note: Immunofluorescence staining was employed to detect the expression of inositol trisphosphate (IP3) in the dorsal spinal horn of mice across different groups. The staining included co-staining with 4',6-diamidino-2-phenylindole (DAPI) for nuclei visualization.

Figure 6. Scratching Times of Mice in Each Group



Note: The figure illustrates the scratching times of mice in each group, demonstrating variations in pruritus behavior.

IP3 Expression in the Dorsal Spinal Horn of Mice in Various Groups

Immunofluorescence staining results indicate elevated IP3 expression in the dorsal spinal horn of mice in the Y group, surpassing levels observed in both the normal and sham groups. Furthermore, comparative analysis reveals that, in comparison to the Y group, IP3 expression increases after intrathecal injection with GRP, while it decreases following intrathecal injection with the GRPR antagonist RC-3095, see Figure 5.

Involvement of GRP/GRPR in the Onset of Uremic Pruritus (UP)

As depicted in Figure 6, scratching times in the Y group decreased compared to both the normal and sham groups. Moreover, when compared to the Y group, scratching times increased over time after intrathecal injection with GRP, while they decreased over time after intrathecal injection with the GRPR antagonist RC-3095, refer to Figure 6.

DISCUSSION

UP is intricately associated with the accumulation of metabolic substances, reduced skin moisture content, disruptions in calcium and phosphorus metabolism, secondary hyperparathyroidism, immune inflammation, inadequate dialysis, and various other factors and mechanisms.[23] This chronic and distressing condition leads to severe discomfort and serves as a significant contributor to uremic mortality.[24]

This disease significantly diminishes patients' sleep quality, giving rise to emotional challenges such as anxiety and depression. Moreover, it often leads to chronic fatigue, exerting a profound negative impact on patients' overall quality of life. Importantly, these factors collectively contribute to a heightened mortality rate among affected individuals.[25] Given these implications, investigating the potential mechanisms underlying UP becomes imperative.

GRPR, situated in laminae I-II of spinal cord neurons, plays an important role in pruritus transmission.[26] On the other hand, GRP, an endogenous pruritus neuropeptide, is expressed in specific cells of the dorsal root ganglion and trigeminal ganglion, activating GRPR in the spinal cord.[20] This complex interaction forms the GRP-GRPR signaling pathway, which is responsible for pruritus transduction.[27] Notably, heightened expression of both GRP and GRPR correlates positively with the intensity of chronic itch, evident in increased scratching bouts among animals.[28] Conversely, a decrease in GRPR or GRP has been observed to alleviate chronic itch. This observation underscores the critical role of GRPR in sustaining chronic itch.[29]

In our investigation, we employed a chronic renal failure mouse model to examine the involvement of gastrin-releasing peptide and its receptor (GRP-GRPR) in the onset of UP. Our findings revealed an elevation in the expression of both GRP

and GRPR in UP, aligning with findings from prior studies.[30] Notably, our study contributes to the understanding that the combination of GRP and GRPR catalyzes the hydrolysis of phosphatidylinositol diphosphate, generating the second messenger's IP3 and diacyl-side oil.[31]

The IP3-$Ca2^{+}$ pathway, initiated by the former mechanism, promotes an increase in intracellular $Ca2^{+}$, a crucial mediator in itch signal transduction.[32] Confirming these findings, our study demonstrated elevated IP3 expression in the dorsal spinal horn of mice in the Y group compared to the normal and sham groups. Furthermore, post intrathecal injection with gastrin-releasing peptide (GRP), IP3 expression increased, while injection with the GRPR antagonist RC-3095 led to a reduction in IP3 expression, emphasizing the intricate modulation of itch signaling in our experimental setting.

Furthermore, studies have reported that mice lacking GRPR exhibit a diminished response to intense itch stimulation, underscoring the pivotal role of GRPR in the itch pathway.[33] Our study's outcomes reinforced this notion, revealing that the GRPR antagonist RC-3095 effectively inhibited scratching behavior induced by pruritus. This observation further substantiates the crucial involvement of GRPR in the intricate network of itch sensation pathways.

Crucially, intravaginal injection of neurotoxin targeted GRPR-positive neurons in the dorsal spinal cord horn, nearly abolishing all scratching responses triggered by pruritus. This compelling evidence strongly implies that GRPR plays a specific and pivotal role in the transduction of neurogenic pruritus signals. Furthermore, our study demonstrated a positive correlation between GRP and GRPR expression and the intensity of chronic pruritus, as evidenced by an observable increase in scratching frequency in the animals. These findings underscore the complex relationship between GRP, GRPR, and the manifestation of chronic pruritus.

On the Contrary, the targeted elimination of the GRPR or *GRP* gene demonstrated a significant alleviation of chronic pruritus, underscoring the pivotal role of GRP/GRPR in its maintenance. This finding aligns with Guo et al.[34] observations, where activation of the GRP/GRPR pathway induced itch-scratching behaviors in mice. Additionally, Norikazu Kiguchi et al.[28] proposal of a cooperative regulation between GRP and glutamate in GRPR+AMPAR+ neurons further supports our conclusion that the GRP-GRPR signaling pathway plays a crucial role in the characteristics of chronic pruritus in UP.

Study Limitations

While our study sheds light on the involvement of the GRP-GRPR signaling pathway in chronic pruritus associated with uremic pruritus, certain limitations warrant consideration. Firstly, the direct determination of GRP and GRPR expression levels in UP patients was not within the scope of this study, leaving a gap in our understanding of the clinical relevance. Additionally, the absence of *in vitro* assays restricts our ability to comprehensively explore the mechanistic intricacies of the GRP-GRPR pathway in UP. Future investigations should address these limitations, incorporating patient data and *in vitro* experiments to provide a more nuanced and comprehensive understanding of the implications of the GRP-GRPR signaling pathway in UP.

CONCLUSION

In conclusion, our investigation highlights the significant involvement of the GRP-GRPR signaling pathway in the onset of uremic pruritus. The elevated expression of GRP and its GRPR in UP mice models underscores the potential relevance of this pathway in the pathogenesis of UP. These findings contribute valuable insights into the molecular mechanisms underlying UP, offering a promising avenue for the development of innovative therapeutic interventions. By identifying the role of the GRP-GRPR signaling pathway, our study lays a foundation for future research aimed at refining treatment strategies and ultimately alleviating the burden of UP in affected individuals.

COMPETING INTERESTS
The authors report no conflict of interest.

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AUTHOR CONTRIBUTIONS
Huili Li and Weiwei Gao contributed equally to this work.

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None.

AVAILABILITY OF DATA AND MATERIALS
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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