Role of the nuclear receptor subfamily 4a in mast cells in the development of irritable bowel syndrome

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ABSTRACT

The activation of mast cells (MCs) and mediator release are closely related to the pathophysiology of irritable bowel syndrome (IBS). However, the exact underlying mechanisms are still not completely understood. The nuclear receptor subfamily 4a (Nr4a) is a family of orphan nuclear receptors implicated in regulating MC activation, degranulation, cytokine/chemokine synthesis and release. Acute and chronic stress trigger hypothalamic–pituitaryadrenal axis (HPA) activation to induce the release of corticotropin-releasing hormone (CRH), resulting in MC activation and induction of the Nr4a family. Our newest data showed that Nr4a members were specially over-expressed in colonic MCs of the chronic water-avoidance stress (WAS)-induced visceral hyperalgesia mice, suggesting that Nr4a members might be involved in the pathophysiology of visceral hypersensitivity. In this review, we highlight the present knowledge on roles of Nr4a members in the activation of MCs and the pathophysiology of IBS, and discuss signaling pathways that modulate the activation of Nr4a family members. We propose that a better understanding of Nr4a members and their modulators may facilitate the development of more selective and effective therapies to treat IBS patients.

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Contents

1. Introduction

Irritable bowel syndrome (IBS) is characterized by chronic or recurrent pain associated with altered bowel motility, with heterogeneous phenotypes ranging from constipation to diarrhea, with a mixed subtype and even an unclassified form, and represents the most important gastrointestinal disorder in humans [1]. Mast cells (MCs) are best known as critical effector cells in many settings of the immune response, such as host defense, immune regulation, allergy, chronic inflammation, and autoimmune diseases through degranulation and release of many biologically active molecules [2,3]. A characteristic feature of MCs is that their population is usually increased in gastrointestinal tract of animal models and biopsies from patients with IBS [4]. Therefore, MC activation and mediator release in proximity to mucosal nerve terminals lead to visceral hypersensitivity, intestinal dysmotility, abnormal intestinal secretion and permeability, which in turn contribute to the development of IBS [5]. However, exact pathophysiological mechanisms are poorly understood. The nuclear receptor subfamily 4a (Nr4a) is highly expressed in activated MCs and acts as transcriptional effectors linking intestinal and non-intestinal disorders such as inflammation, allergy, autoimmune diseases and carcinogenesis [6,7]. Members of the Nr4a family have been shown to have a regulatory role in MC activation, degranulation and release of many active mediators [7]. Our recent study found that Nr4a members were specially overexpressed in colonic MCs of the chronic water-avoidance stress (WAS)-induced visceral hyperalgesia mice, suggesting these receptors might be involved in the pathophysiology of visceral hyperalgesia [8]. In this review, we focus on the role of Nr4a members in MC activation, gut function and the immune response, and its involvement in IBS pathogenesis, especially that associated with stress, and discuss signaling pathways that modulate the activation of Nr4a members.

2. The activation of MCs is involved in the development of IBS

As an essential part of the enteric immune system, MCs may be sensitized and activate the inflammatory cascade in response to both extrinsic (bacteria, fungi, viruses, parasites and diet) and intrinsic factors (neurotransmitters, cytokines and hormones) [9]. MCs express the high-affinity receptor for immunoglobulin E (IgE), the Fc epsilon RI (FcεRI) in the cell-surface. FcεRI cross-linking, e.g. by an allergen, results in the immediate release of preformed mediators (such as histamine, proteases, and cytokines) by degranulation and de novo production of lipid mediators and cytokines by the activation of transcriptional events, e.g. mediated by nuclear factor of activated T-cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and activator protein 1 (AP-1) [10]. According to their sites of location and repertoire of expressed proteases, MCs are broadly divided into mucosal and connective tissue types [11]. In gastrointestinal tract, MCs are widely distributed in the mucosa, submucosa and lamina propria, where pathogens, allergens and other environmental agents may be encountered [11]. In these locations, MCs are mainly seen surrounding blood vessels, enteric neurons or nerve fibers [12,13], suggested the anatomical and functional associations between MCs and nervous system [11]. The functional consequence of this close anatomical link is that diverse neurons and MCs within the gut act as a surveillance network.

Functional evidence suggested that MCs are involved in the interaction between immune cells and neurons in the gut, thus being considered the major effector cells for immediate hypersensitivity for the maintenance of the gastrointestinal tract homeostasis [14]. Neuroimmune interactions in the gut consist of different components, such as intestinal MCs, enteric neurons, visceral afferent and efferent fibers that form an interaction network [15]. In such close contact between neuronal cells and MCs, MCs function as both sensory and effector cells in communication [14]. MCs are activated by cytokines and by neuropeptides such as substance P (SP), vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) [16,17]. Once activated, MCs release inflammatory mediators such as tryptase, chymase and proinflammatory cytokines leading to inflammation and pain hypersensitivity [14]. The cytokines/inflammatory factors secreted by MCs have a bidirectional communication between neural signal processing and immune response, which are an important bridge between the nervous and immune systems [18]. The abnormal release of mediators by intestinal MCs has been suggested to be involved in visceral hypersensitivity and abdominal pain in patients with IBS [19]. In addition to the increase of intestinal MCs in patients with IBS, our previous study also showed that many inflammatory mediators, such as interleukin (IL)-1β, inducible nitric oxide synthase (iNOS) and P2X purinoceptor 7 (P2X7) increased in the colonic MCs of IBS patients [20]. Cytokine-chip data showed that the development of IBS and visceral hypersensitivity are closely related to changes in the cytokine/chemokine expression profile of MCs [21,22]. Furthermore, tryptase released by MCs promote the formation of peripheral sensitization through activating protease-activated receptor 2 (PAR2) located on enteric nerves and visceral afferents [23]. In this sense, MCs play an important role in the activation of enteric neurons in the gut and maintain the hyperexcitability of nociceptors in the development of IBS. In addition, more recently, a role of MCs has also been hypothesized in relation to IBS-like disorders, such as gluten sensitivity and adverse reaction to foods with a high nickel content [24,25].

Accumulating evidence showed that Nr4a family members are highly expressed in activated murine and human MCs through FcεRI cross-linking and other various MC-activating stimuli [26,27]. Notably, the induction of Nr4a members have been shown to promote the production of cytokine/chemokine and degranulation in activated MCs via FcεRI cross-linking [7]. Recent study indicated that their high expressions in colonic MCs was associated with visceral hyperalgesia in WAS mice [8]. These findings collectively provide a molecular mechanism that connects these receptors with activation of MCs, which release many biologically active mediators, and subsequent visceral hyperalgesia and other symptoms of IBS.

3. Structural and functional characteristics of the Nr4a family

The Nr4a is a family of orphan nuclear receptors because the endogenous ligands of these receptors do not have well-characterized [28,29]. They belong to the larger nuclear receptor superfamily of eukaryotic transcription factors [28]. The Nr4a subfamily contains three highly homologous members named Nr4a1 (also known as Nurr77, nerve growth factor-induced clone B, NGFI-B), Nr4a2 (also known as Nurr1) and Nr4a3 (also known as neuron-derived orphan receptor 1, NOR1) [30]. All three Nr4a members consist of a ligand independent activation function-1
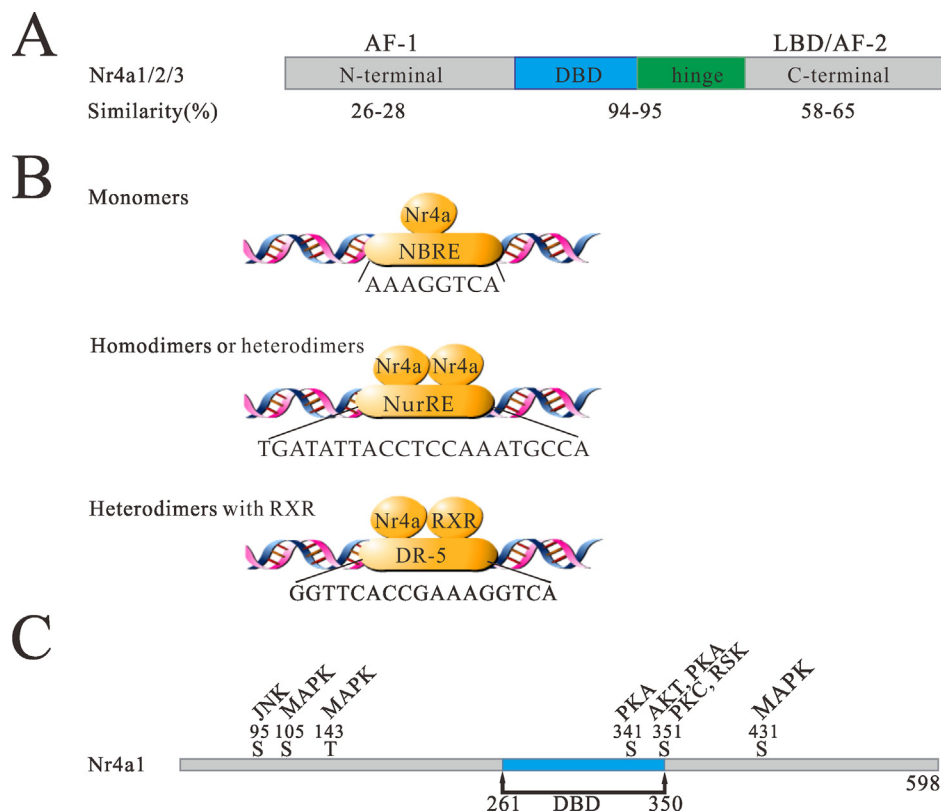
Fig. 1. Nr4a family members share common structure/function domains and are modulated by kinases. (A) Domain structure of Nr4a orphan nuclear receptors and similarities among Nr4a1, Nr4a2 and Nr4a3. Nr4a members contain a variable N-terminal region (AF-1), a conserved DNA-binding domain (DBD), a hinge region and a variable C-terminal region (LBD/AF-2). (B) Specific binding sequences for the Nr4a subfamily. Nr4a family members act as transcription factors by regulating the transcription of their target genes through binding as monomers to NBRE sequences, as homodimers or heterodimers to NurRE sequences, or heterodimers with RXR to DR-5 sequences. (C) Overview of the amino-acid sequence of Nr4a1 with known phosphorylation sites and associated kinases indicated (T = threonine, S = serine).

(AF-1) transactivation domain in the N-terminal region, a highly conserved DNA-binding domain (DBD) composed of 2 zinc fingers recognizing specific DNA sequences, a hinge region and a ligand-binding domain (LBD) that contains a ligand dependent AF-2 trans-activation domain in its C-terminal portion [31,32] (Fig. 1A). The AF-1 domain that serves as a ligand-independent transcription activation domain is required for the recruitment of other transcription factors and transcriptional activity in the N-terminal region [33].

Nr4a orphan nuclear receptors function as ligand-independent transcription factors and immediate- or early-response genes, which are rapidly up-regulated by a variety of stimuli (such as stress, cytokines, infectious factors, and growth factors) [32]. Once activated, these Nr4a members act as transcription factors by binding to specific sequence sites in the promoter region of their target genes [31] (Fig. 1B). Specifically, Nr4a members induce gene expression by binding as monomers to the NGFI-B response element (NBRE, sequence: AAAGGTCA), and as homodimers or heterodimers to the Nur response element (NurRE, sequence: TGATATTACCTCCAAATGCCA) in the promoter of their regulated target genes [34]. Furthermore, Nr4a1 and Nr4a2 (but not Nr4a3) can heterodimerize with the retinoid X receptor (RXR), and activate transcription of a response element with a five-nucleotide spacer (GGTTCACCGAAAGGTCA) (direct repeat 5, DR-5) in a 9-cis-retinoic acid (9cRA)-dependent manner [35]. Though ligands for these orphan nuclear receptors are presently undiscovered, they are thought to function as constitutively active and ligand-independent receptors, whose transcriptional activity is primarily dependent on the expression of receptors and their posttranslational modifications [36–38]. However, a few ligands that may

interact with these receptors are discovered in previous reports [36–38]. Therefore, it is not known whether the Nr4a subclass still belongs to the ligand-independent orphan nuclear receptor family. More experimental results are needed to provide more definitive conclusions. Furthermore, different Nr4a members can form heterodimers and synergistically regulate expression of genes [39]. In addition, RXR has been shown to increase the expression and activity of Nr4a orphan nuclear receptors, which was enhanced further upon addition of the RXR ligand 9cRA [40]. This interaction can further enhance the formation of RXR and therefore make Nr4a indirectly linked to retinoid retinoic acid signaling [41].

In addition to the rapid and transient Nr4a expression, the transcriptional activity of these orphan nuclear receptors is regulated by posttranslational protein modification of the receptor including phosphorylation [42]. It has been reported that all three Nr4a members can be phosphorylated at serine or threonine residues by growth factor-dependent activation of protein kinases, such as mitogen-activated protein kinase (MAPK), phosphatidyli-nositol 3-kinase (PI3K), serine/threonine-specific protein kinase AKT (AKT), Jun-N-terminal kinase (JNK), and ribosomal S6 kinase (RSK) [42] (Fig. 1C). For example, Nr4a1 is thought to play a role in transcriptional regulation through the phosphorylation at serine 350 and serine 354 within the DNA-binding domain, which suppresses the transactivation activity [43,44]. Moreover, the phosphorylation of Nr4a1 at serine 105 results in translocation of the Nr4a1/RXR heterodimer complex out of the nucleus [45], providing an additional mechanism by which the phosphorylation may inhibit the transcriptional activity of Nr4a1. The posttranslational modifications that regulate these events are of utmost importance to understand the functional role of Nr4a members

and may represent a major mode of the control of gene expression by Nr4a members.

In recent decades, studies have shown that members of the Nr4a subfamily are involved in a wide range of disorders including cancer, inflammation, neurological or cardiovascular diseases, and immune alterations, and plays a key role in the physiological and pathological processes of cells [46]. In response to inflammatory reaction, all three Nr4a members are strongly induced by inflammatory stimuli in macrophages [47]. It has been shown that Nr4a members are strongly upregulated in macrophages by a series of proinflammatory stimuli, such as lipopolysaccharide (LPS), cytokines and oxidized lipids, thereby suggesting that these receptors are involved in the transcriptional regulation of inflammatory response [48,49]. Although Nr4a orphan nuclear receptors were originally described as only activate genes, previous study supplied the evidence that some of these members can also suppress the production of proinflammatory mediators in response to inflammatory stimuli by recruiting corepressor for element-1-silencing transcription factor (CoREST) complex to NF-kB target genes [50,51]. For example, stimulation of Nr4a subfamily members such as Nr4a1 and Nr4a2 regulated microglial functions, inhibited the production of several inflammatory factors such as IL-1β, CCL2 and CXCL2, and alleviates neuropathological events in several disease models [52]. Furthermore, recent evidence showed the Nr4a subfamily as a key transcription factor for immune tolerance that suppresses cytokine expression and induces various immunoregulatory molecules including the suppressor of cytokine signaling in CD4+ T cells, CD8+ T cells, and regulatory T (Treg) cells [53]. Moreover, the Nr4a subfamily also participates in the regulation of autoimmunity and infection-related immune responses by engaging on Treg cells differentiation and induces the CD8+ T cell dysfunction program by strengthening NFAT signaling [54].

4. The induction of the Nr4a family following the activation of MCs

As a molecular switch for gene regulation, the Nr4a family plays an important role in the integration of complex cytokine signaling pathways by acting as downstream mediator of inflammatory signaling pathways [55,56]. Previous report showed that Nr4a family members of mouse bone marrow-derived MCs (BMMCs) are important regulators of MCs function [57]. The activation of BMMCs and human MCs by IgE receptor cross-linking induced all three Nr4a members over-expression, and IgE receptor cross-linking and calcium ionophore stimulation of BMMCs and LAD2 cells induced Nr4a phosphorylation respectively [27]. Co-culture of BMMCs with bacteria resulted in a remarkable and selective over-expression of all three Nr4a members [27]. Treatment of MCs with purified LPS can selectively up-regulate Nr4a3, but have no significant effect on the expression of Nr4a1 or Nr4a2 [27].

Computational approaches utilizing next-generation sequencing data offer further understanding of the expression of Nr4a
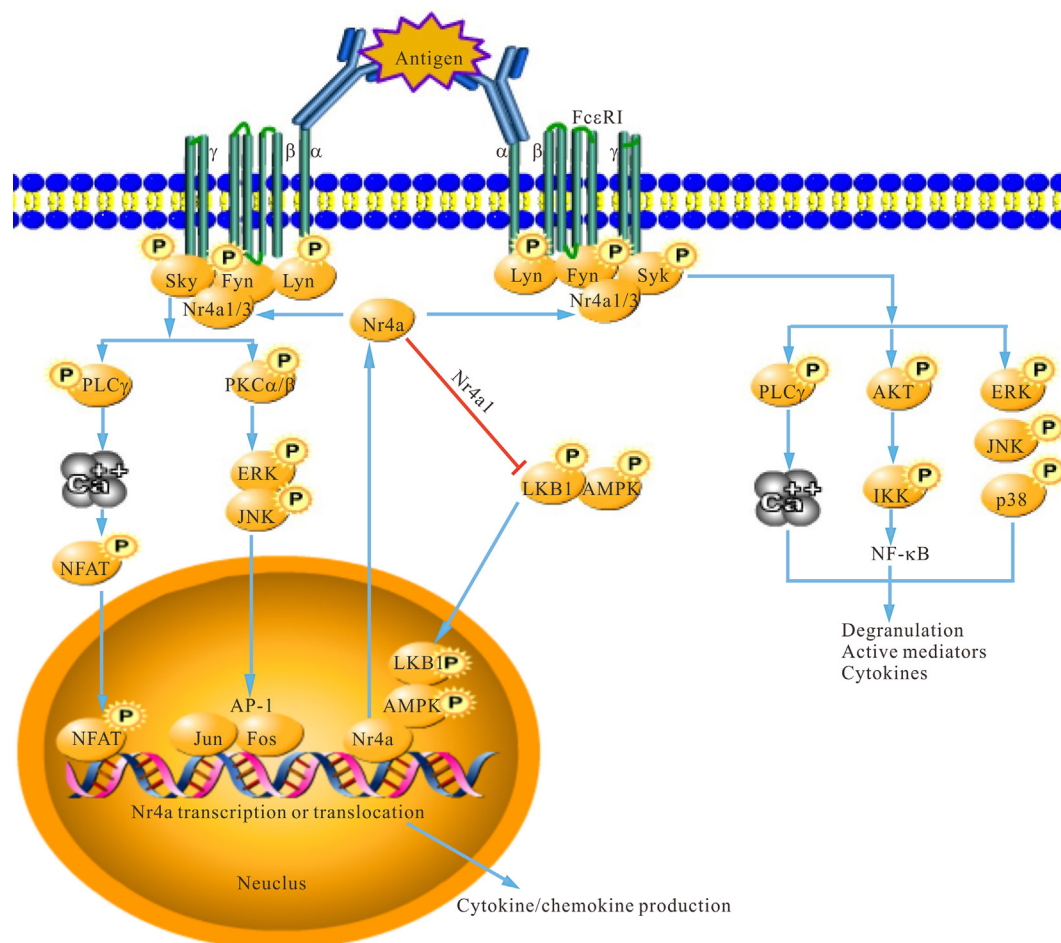
Fig. 2. Summary of the possible mechanisms of the Nr4a subfamily in FcεRI-mediated MC activation. The Nr4a gene expression or nuclear translocation is induced by FcεRI cross-linking in activated MCs via the PKC/ERK pathway and the calcineurin-NFAT pathway, which provokes cytokine/chemokine gene transcription. Furthermore, Nr4a1 could promote MC activation by inhibiting the LKB1/AMPK signal pathway following FcεRI cross-linking. In addition, Nr4a1 NR4a3 could also positively regulate MC activation and degranulation by facilitating both Syk and Fyn activations.

family member at the genomic level following MC activation. Recently, our RNA Sequencing (RNA-Seq) results showed that MCs (P815) treated with CGRP significantly up-regulated Nr4a2 and Nr4a3 gene expression [8]. Similar to what we observed in MCs induced by CGRP, a Functional Annotation of Mammalian Genome 5 (FANTOM5) Consortium data reported that Nr4a1 with highest activity was identified in Langerhans cells and MCs across 1,900 samples from across the body, while both Nr4a2 and Nr4a3 were induced in human MCs stimulated by FcεRI cross-linking [57]. In another Immunologic Genome (ImmGen) Consortium paper for the mouse, both Nr4a2 and Nr4a3 were induced in peritoneal MCs by enzymatic treatment [58]. However, quantitative proteomics showed that there was no significant difference in protein expression of three Nr4a members between human MCs and peripheral blood mononuclear cells (PBMCs) [59]. The inconsistency between transcriptome profiles and quantitative proteomics may be a result of the MC subset employed, because in vitro MCs stimulated by FcεRI cross-linking and enzymatic treatment of MCs showed increased expression of Nr4a members.

By using specific inhibitors of signaling pathways, Nr4a3 expression in BMMCs was shown to be strongly dependent on Gö6976-sensitive protein kinase C (PKCα) and partially dependent on the calcineurin/NFAT signaling [27]. However, the treatment with inhibitor of NF-kB failed to abolish the expression of Nr4a3 in BMMCs [27]. Among several pathways downstream of the Ca^{2+} influx enhanced by MC activation, the pathway of serine/threonine phosphatase calcineurin and its target transcription factor NFAT has a major role in the transmission of calcium signals from the cytosol into the nucleus to interact with response elements and induce the expression of all three Nr4a members and a variety of cytokines [60]. Previous report has identified a link between the Nr4a family and adenosine, a paradoxical inflammatory molecule that can contribute to persistence of inflammation or mediate inflammatory shutdown via PKC/extracellular regulated protein kinases (ERK) signal pathways in MCs [46]. Furthermore, exposure of MCs to gram-positive Streptococci resulted in a dramatic upregulation of all three Nr4a members and generated cytokine responses through toll-like receptor 2 signal [26]. Together these data illustrate that signaling pathways such as PKCα, ERK, NFAT have been proven to be activated after FcεRI triggering and those pathways that, hypothetically, could lead to the activation of Nr4a at the transcriptional level (increase on Nr4a mRNA) or at the level of nuclear translocation (Fig. 2).

5. The role of the Nr4a family in the function of MCs

Although it has been confirmed that over-expression of Nr4a members in activated MCs resulted in degranulation [27], but the

mechanism is not clear. The selective expression of Nr4a members in activated MCs by various stimuli implicated these orphan nuclear receptors may have different roles in gene regulation. Further studies showed that Nr4a3 is a transcription factor for IL-6, IL-13, monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-α production in MC and the major pathway involves the transcription factor belonging to the NFAT family [27,60]. This suggests that Nr4a3 may have a proinflammatory role in terms of regulating cytokine/chemokine responses in a MC setting [46]. These findings are in line with previous studies in which Nr4a family members have been implicated in the regulation of inflammatory gene expression in macrophages activated through pattern recognition receptors [47,48]. Moreover, Nr4a3 can further regulate FcεRI-mediated degranulation, and negatively regulate the tryptase gene and protein expression of MC as well as the responsiveness in allergen-induced MC activation [27,46]. In addition, Nr4a2 may have a role in activation-induced cell apoptosis in MCs, as shown in growth factor deprivation-induced cell apoptosis of BMMCs [27]. These evidences indicated that Nr4a members are the functional regulator related to the homeostasis and activation of MCs by various stimuli.

It has been clearly shown that FcεRI cross-linking causes activation of components of the inducible kinase inhibitor of nuclear factor kappa-B kinase (IKK) complex and Lyn and Fyn pathways [61]. The NF-kB signal pathway has been implicated in FcεRI cross-linking induced production of proinflammatory cytokines in MCs [61]. IKKα and IKKβ have been shown to be important for cytokine production in MC activation following FcεRI cross-linking [62]. It has been confirmed that the Nr4a family can influence NF-kB pathway by regulating the expression of IKK components in macrophages [47,48]. The earliest event in response to FcεRI cross-linking in MCs is the activation of two Src-family protein tyrosine kinases Fyn and Lyn, which phosphorylate the immunoreceptor tyrosine-based activation motifs of the β and γ chains of FcεRI [62,63]. Both Lyn and Fyn are considered key components of the signal transduction cascade resulting in degranulation and the synthesis of IL-1β, IL-10, and MCP-1 in MCs [63,64]. To date, no direct connection between those events and the activation of Nr4a has been described. Although FcεRI cross-linking causes activation of components of IKK complex, Lyn and Fyn signals in MCs, whether those events mediated the activation of Nr4a members needs to be further studied. However, previous study showed that Nr4a3 knockout was associated with down-regulated Fyn expression whereas Lyn was unchanged in MCs, suggesting Nr4a3 up-regulated Fyn expression [27]. Moreover, Nr4a1 could promote MC activation and degranulation by inhibiting the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) signal following FcεRI cross-linking [65] (Fig. 2). In addition, Nr4a1 could also

Table 1
Upstream and downstream events in Nr4a-mediated signaling in MC activation.

Events in Nr4a activation	Upstream/downstream	Function/targets	References
PKC signaling pathway	Upstream	↑ Nr4a2,3 expression	[27,46]
ERK1/2 signal pathway	Upstream	↑ Nr4a2,3 expression	[46]
Calcineurin/NFAT pathway	Upstream	↑ Nr4a1-3 expression	[27,60]
Toll-like receptor	Upstream	↑ Nr4a1-3 expression	[26]
FcεRI cross-linking	Upstream	↑ Nr4a1-3 expression	[7,46,57,60]
G-coupled receptors	Upstream	↑ Nr4a1-3 expression	[8,46]
Calcium ionophore	Upstream	↑ Nr4a1-3 expression	[7,46,60]
Nr4a2 expression	Downstream	↑ MC apoptosis	[27]
Nr4a1-3 expression	Downstream	↑ cytokine/chemokines	[46,27,60,65]
Nr4a3 expression	Downstream	↓ tryptase expression	[7]
Nr4a3 expression	Downstream	↑ Fyn expression	[7]
Nr4a1 expression	Downstream	↑ Syk activation	[65]
Nr4a1 expression	Downstream	↓ LKB1/ AMPK signal	[65]
Nr4a1-3 expression	Downstream	↑MC activation and degranulation	[7,46,27,60,65]
Nr4a3 expression	Downstream	↓MC's responsiveness to allergen	[7]

positively regulate MC activation by facilitating Syk activation [65]. The upstream and downstream events in Nr4a-mediated signaling in MC activation are shown in Table 1.

6. The role of the Nr4a family in the development of IBS

The Nr4a family plays a prominent role in MCs-mediated immune and inflammatory response, and are also involved in a variety of gastrointestinal and non-gastrointestinal disorders such as inflammation, allergy, autoimmune diseases and carcinogenesis [6,66,67]. Nr4a members have been identified as important nuclear factors linking chronic inflammation and carcinogenesis in the gut, especially colorectal carcinoma cells [67]. Evidence has shown that Nr4a2 is upregulated in psoriatic arthritis synovium and psoriatic skin by corticotropin-releasing hormone (CRH) signal [66]. Deficiency of Nr4a family members inhibited the IgE/antigen-induced upregulation of TNF-α mRNA expression in mucosal MCs through calcineurin activity [60]. These findings collectively suggest a significant role for Nr4a-induced MC activation in immune-mediated inflammatory disease.

The hypothalamic–pituitaryadrenal (HPA) axis is a complex feedback system of neurohormones comprising of the hypothalamus, pituitary and adrenal glands [68]. This negative and positive feedback system controls reactions to stress and has important roles in regulating various body processes such as digestion and the immune response [68]. Visceral hyperalgesia and HPA axis dysregulation are common features in patients with IBS [11,68]. CRH is a primary activator of the HPA axis, integrating effects of stress through HPA axis activating with subsequent regulation of several gastrointestinal functions, such as motility, sensitivity, secretion, absorption, microcirculation in the gut, and local immune responses [68]. HPA axis components including CRH and its receptors (CRH-R) are widely expressed in the gastrointestinal tract, and display different CRH-R subtypes according to cell type, physiological fluctuations and disease status [69]. This indicates that the gastrointestinal tract has a gut HPA-like system that regulates local

functions. CRH is a major mediator of stress response in IBS pathogenesis and has been proposed to contribute to the visceral allodynia and increased colonic permeability in pathophysiology of IBS through activating intestinal mucosal MCs [70]. MCs are highly responsive to activation of the HPA axis as they express the CRH-R1 [69]. CRH operates via its receptor that, in intestinal MCs, can lead to release inflammatory chemical mediators, and cytokines/chemokines, which are the most potent stimulators of HPA axis [71]. Increasing evidence supports that peripheral CRH and its receptor play important roles in the colonic response to stress and brain-gut sensitization [72]. Studies in animals suggest that CRH can promote visceral hypersensitivity and increase colon permeability by activating MCs in the intestinal mucosa that associated with the pathogenesis of IBS [72]. It has been identified that the expression of CRH-R1 in the intestinal MCs of patients with IBS is significantly increased and mediates the local intestinal stress (visceral hypersensitivity) response [73]. Therefore, CRH, produced locally or released by peripheral nerves, may be a key component of HPA axis mediating interactions between the central and gastrointestinal stress response.

Several lines of evidence indicate that all three Nr4a members play an important coordinate regulatory role at all levels of the HPA axis [74–76]. Previous studies have shown that psychological stress induces Nr4a2 expression in various brain tissues, and Nr4a3 is usually used as the marker of neuronal activation after a stressful stimulus [74,76]. The expression of Nr4a1 and Nr4a3, as well as CRH genes was found to be upregulated in the hypothalamus after stress [73]. A functional NurRE motif has been demonstrated naturally occurring in the pro-opiomelanocortin (PomC) gene promoter in primary pituitary cells [77]. Nr4a1 and Nr4a2 have been identified to be main mediators of CRH/cAMP stimulation of the adrenocorticotropin hormone (ACTH) precursor (PomC) gene expression in pituitary corticotrophic cells by binding to two sites in the PomC promoter, NBRE and NurRE sequences, thereby countering actions of glucocorticoids [43,78]. In the adrenal gland, restraint stress also had marked effects on the adrenocortical mRNA expression of all three Nr4a members, steroidogenic factor 1 (Nr5a1) and steroid

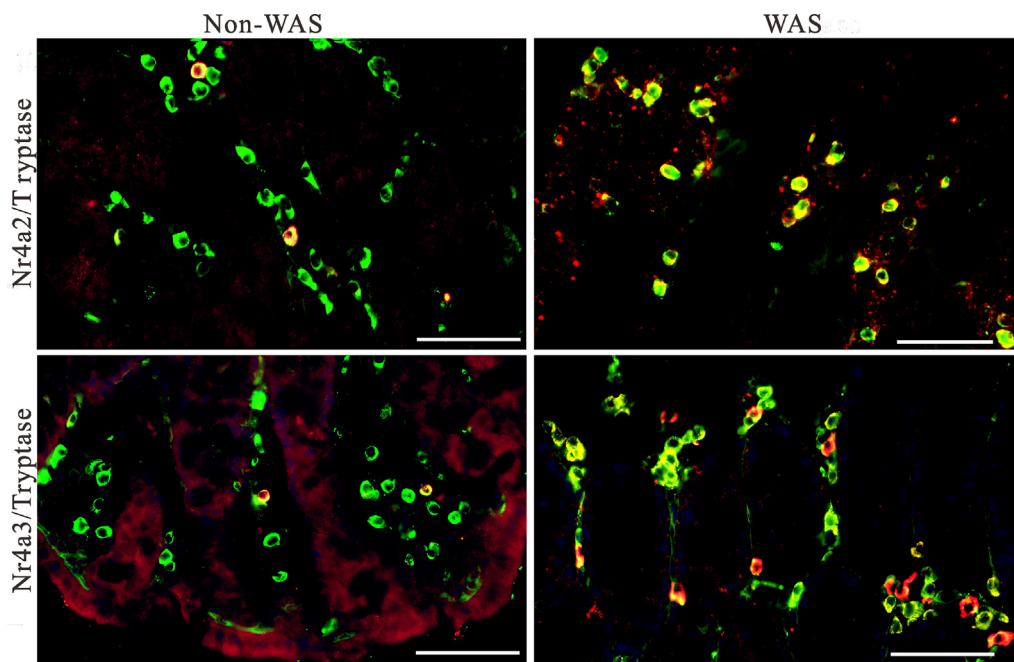**Fig. 3.** Double immunofluorescence staining with Nr4a2 (red) or Nr4a3 (red) and tryptase (a marker of MCs, green) revealed extensive co-localization (yellow) of Nr4a2 or Nr4a3 and tryptase in the colonic MCs of WAS mice, compared with non-WAS mice (normal). Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

21-hydroxylase [73]. Studies have shown that all three Nr4a members have a rapid stimulatory effect on steroidogenesis by increasing several steroidogenic enzymes through binding to NBRE in the promoter region, e.g., 21α-hydroxylase and 3β hydroxysteroid dehydrogenase [79]. Moreover, all three Nr4a members are rapidly and transiently induced after CRH treatment to activate the HPA axis with upregulated ACTH and cortisol production [80,81]. These findings suggest that transcription factors Nr4a members may be involved in CRH-mediated stress response through HPA axis. In addition to mediated local stress response by activated MCs, CRH also induces the expression of Nr4a2 and Nr4a3 [8,66] in activated MCs, which emerges as an important nuclear factor modulating visceral hyperalgesia and gastrointestinal inflammation [8,67]. Therefore, the activation of MCs and the expression of Nr4a members induced by CRH may mediate interactions between the HPA axis and gastrointestinal tract - the "brain-gut axis" in IBS pathogenesis.

The intestinal microbiome, key components of the gut-brain axis, impacts health and disease in several ways by influencing the immune, endocrine, and central nervous systems [82]. Chronic stress may cause an imbalance in bacterial communities, or dysbiosis in the gut [83]. And conversely, gut dysbiosis may activate stress responses that activate MCs with release of many biologically active molecules of potential relevance to the pathophysiology of IBS [4]. Previous reports have shown that gut microbes, such as bacteria and fungi, can induce MC activation through the recognition receptors on the cell surface [84]. Studies in vitro have indicated that exposure of murine MCs to live bacteria promotes a selective expression of all three Nr4a members [27]. So far, however, no direct connection between gut dysbiosis and the expression of Nr4a members in MCs has been described. Although antibiotics-driven gut microbiome perturbation enhanced Nr4a expression and increased immune cell activation in the gut [85], whether the gut dysbiosis mediated the expression of Nr4a members in activated MCs needs to be further studied.

7. Stress-induced colonic MC activation selectively upregulates Nr4a members

The psychosocial stress-related regulation of visceral hyperalgesia to intestinal stimuli has been well described in preclinical mouse models of IBS [8,86]. The well-characterized chronic WAS represents a potent psychological stressor with significant increases of stress hormones, intestinal MCs activation and infiltration, and visceral hyperalgesia [87,88]. Increasing evidence has indicated that Nr4a members play a crucial role in MC degranulation and cytokine generation [46], which lead to visceral hypersensitivity in IBS pathogenesis. The role of these receptors was further established to mediate visceral hypersensitivity induced by stressor in mouse using WAS approach [8]. Our recent study demonstrated that the increased colonic MCs in WAS mice specially over-expressed Nr4a2 or Nr4a3 (Fig. 3). The majority of mucosal Nr4a3-staining MCs were found in close proximity to the CGRP-immunoreactive nerve fibers in the colon of WAS mice [8]. MCs (P815) treated with CGRP significantly up-regulated Nr4a2 and Nr4a3 gene expression and promoted MC proliferation, while Nr4a3 knockdown may attenuate the promotion effect of CGRP on MC viability [8]. Taken together with previous research, this indicates that the up-regulation of Nr4a members in MCs might not only be induced by FcεRI cross-linking signaling [46], but also be increased by the release of CGRP from enteric neurons and afferents in the gastrointestinal tract. Furthermore, transcriptomic analysis of MCs treated with CGRP identified that differentially expressed genes most enriched in response to CRH and MC activation [8], which are associated with stress-induced visceral hyperalgesia in IBS pathogenesis [68]. The close anatomical relationship between selective over-expression of Nr4a members in colonic MCs and increased CGRP-immunoreactive fibers in the colon of WAS mice allow for the hypothesis that expression of Nr4a members in MCs may be associated with the generation and maintenance of visceral hypersensitivity in IBS patients. In addition,
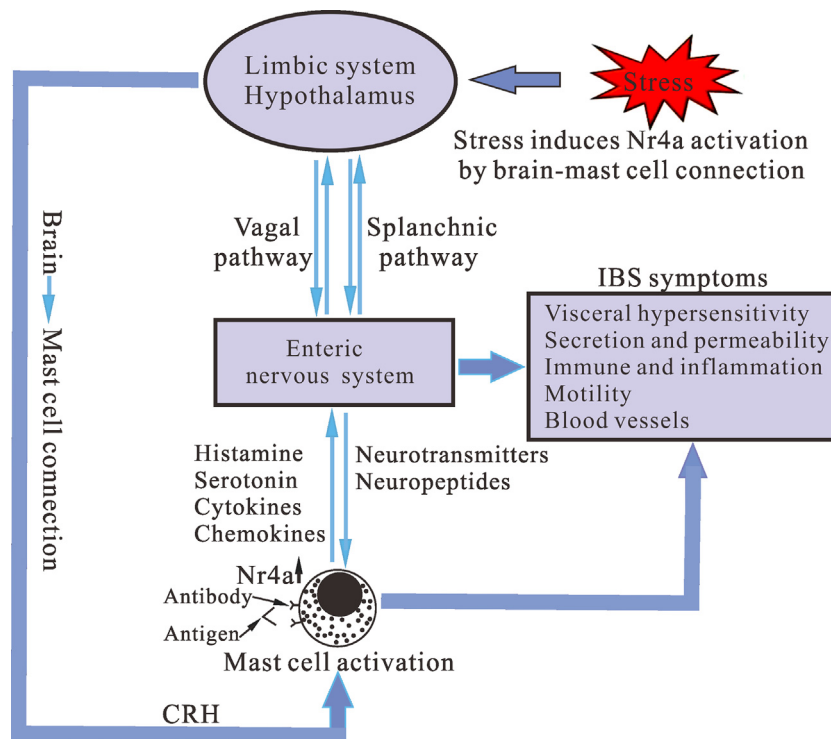
Fig. 4. Summary of the possible role of the Nr4a subfamily in the development of IBS through brain-MC connection activation by stress. The exposure to stress leads to CRH release from HPA axis, which induced gastrointestinal MC activation and Nr4a expression, as well as subsequent the changes in visceral sensitivity, gastrointestinal secretion and permeability, local immune response and inflammation, gastrointestinal motility and local blood flow.

mucosal MCs play an essential role in the development of IBS-like disorders (such as food allergy) in a murine model [25]. Shikonin, a constituent of Lithospermum erythrorhizon exhibits anti-allergic effects by suppressing Nr4a family gene expression as a new prototype of calcineurin inhibitor in mucosal MCs in food allergic disease [60].

CGRP that is a potent modulator involved in immune responses and pain transmission plays an important role in interactions between the nervous system and the immune system during chronic stress [89]. The release of CGRP from sensory nerve terminals can cause MC activation and release tryptase [89–91], which is similar to the degranulation of MCs induced by calcium ionophore in many ways [91]. In addition to its role as a potent modulator of pain, CGRP is involved in a various of stress responses, including activation of the HPA axis by increased the CRH expression in the paraventricular nucleus (PVN) and central nucleus of the amygdale [92,93]. CRH that acts as a major mediator of stress response [66] can also regulate the release of CGRP from nociceptive nerve endings and participate in the regulation of immune response and inflammation [94,95]. Furthermore, CGRP acting on its receptor complex in the bed nucleus of the stria terminalis regulates behavioral stress responses through increasing release of CRH from these neurons [96], suggesting a functional link between CGRP and CRH in stress responses. Because the stress-induced visceral hypersensitivity associated with the specially over-expressed Nr4a members in colonic MCs, it is likely that activation of these orphan nuclear receptors mediated the visceral hypersensitivity and development of IBS.

Exposure to stress evokes persistent alterations in the brain-gut interactions ("brain-gut axis") ultimately leading to the alterations in gastrointestinal motility, increase in visceral perception, and changes in gastrointestinal secretion, etc. [97–99]. Intestinal MCs can respond to stress by communicating with the gut-brain axis that translate stress signals into the release of a wide range of neuromodulators and inflammatory mediators, which may profoundly influence the gastrointestinal function [98]. Morphological evidence suggests that enteric MCs are innervated by efferent fibers from the central nervous system and can be activated by neurons releasing CRH [99]. Functional evidence supporting the brain-MC connection is revealed in results of Pavlovian conditioning of enteric MC degranulation in the gastrointestinal tract [99]. Therefore, an induction of Nr4a members by the brain-MC connection is the most likely mechanism of symptom exacerbation by psychogenic stress in patients with IBS [99] (Fig. 4). This hypothesis is supported by the result that CRH may influence MC activation and the induction of Nr4a members [8,66]. However, an association between the activation of the brain-gut axis, CRH production and an increase on immunoreactivity of Nr4a members in MCs does not necessarily imply cause-effect. Further research is needed to shed more light on this occurrence.

8. Summary and outlook

Compelling evidence described in this review suggests a potential role for the Nr4a subfamily in the regulation of MC activation, cytokine/chemokine generation and the development of IBS. Taken together, these observations identify the Nr4a subfamily as potential molecular targets for IBS and IBS-like disorders (such as food allergy) treatment. Preliminary immunofluorescence results confirm that Nr4a members are selectively over-expressed in the colonic MCs of stress-induced visceral hyperalgesia mice. However, the increase on the expression of Nr4a members in MCs in the analyzed conditions is, for sure, relevant, but evidence indicating their role on MCs-dependent inflammation is still observational. Further studies should be performed to clarify the exact role of these

nuclear receptors in the gastrointestinal MCs and the pathophysiology of IBS.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Competing Interest

The authors do not have any conflict of interest to declare.

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