Lack of Galanin Receptor 3 Alleviates Psoriasis by Altering Vascularization, Immune Cell Infiltration, and Cytokine Expression

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The neuropeptide galanin is distributed in the central and peripheral nervous systems and in non-neuronal organs, including the skin. Galanin acts via three G protein-coupled receptors which, except galanin receptor 1, are expressed in various skin structures. The galanin system has been associated with inflammatory processes of the skin and of several other organs. Psoriasis is an inflammatory skin disease with increased neovascularization, keratinocyte hyperproliferation, a proinflammatory cytokine milieu, and immune cell infiltration. In this study, we showed that galanin receptor 3 is present in endothelial cells in human and murine dermal vessels and is co-expressed with nestin in neo-vessels of psoriatic patients. Moreover, in a murine psoriasis model, we showed that C57/BL6 mice lacking galanin receptor 3 display a milder course of psoriasis upon imiquimod treatment, leading to decreased disease severity, delayed neo-vascularization, reduced infiltration of neutrophils, and significantly lower levels of proinflammatory cytokines compared with wild-type mice. In contrast, galanin receptor 2-knockout animals did not differ significantly from wild type mice at both the macroscopic and molecular levels in their inflammatory response to imiquimod treatment. Our data indicate that galanin receptor 3, but not galanin receptor 2, plays an important role in psoriasis-like skin inflammation.


INTRODUCTION

The mammalian skin forms the largest organ of the body and includes a network of cutaneous nerves, cells of the immune system, and mediators of the neuro-endocrine system (Theoharides et al., 2016). Analogous to the hypothalamic-pituitary-adrenal axis, neuropeptides and their receptors are present in the skin, including galanin (GAL) (Lang et al., 2015; Theoharides et al., 2016).

GAL is a neuropeptide with a length of 30 amino acids (29 in rodents) that is distributed in the central and peripheral nervous systems and in non-neuronal organs such as the skin (Lang et al., 2015). GAL is localized in afferent sensory neurons and specialized cutaneous sensory structures such as Merkel cells and Meissner corpuscles and in non-neuronal structures like eccrine sweat glands, in follicular and epidermal keratinocytes, and in smooth muscle cells of dermal blood vessels (Fantini and Johansson, 1995; Johansson et al., 1999; Johansson et al., 1988; Kofler et al., 2004; Pincelli et al., 1990; Xu et al., 1991). In addition, in a murine model of allergic contact dermatitis, a significant elevation of GAL-positive nerve fibers was observed compared with healthy skin. However, the concentration of the peptide, as measured by radioimmune assay of skin extracts, was markedly reduced (El-Nour et al., 2004). Carrageenan-induced inflammation in the rat hind paw led to elevated GAL-like immunoreactivity and GAL mRNA in the epidermis, as well as GAL-like immunoreactivity in ED-1-positive immunocytes in the dermis (Ji et al., 1995).

GAL exerts its functions via three G protein-coupled receptors (GAL1-R, GAL2-R, and GAL3-R) (Fathi et al., 1997; Habert-Ortoli et al., 1994; Howard et al., 1997). The first evidence for GAL receptor expression in human skin was provided by receptor autoradiography, which detected GAL binding around dermal blood vessels and sweat gland cells (Kofler et al., 2004). Analysis of human skin showed GAL2-R-like immunoreactivity in the basal layer of the epidermis, around dermal blood vessels, and in sweat gland cells (Bovell et al., 2012;
Dallos et al., 2006). GAL3-R—like immunoreactivity was detected in sweat gland cells, and GAL3-R mRNA was shown to be expressed by outer root sheath keratinocytes and hair follicles (Bovell et al., 2012; Holub et al., 2012). In addition, GAL2-R and GAL3-R are expressed on immune cells such as neutrophils, macrophages, and monocytes, which are, inter alia, important in maintaining skin immunity (Chiu et al., 2013; Locker et al., 2015). In contrast, there is no evidence so far for GAL1-R expression in human skin (Bovell et al., 2012; Dallos et al., 2006). It is well known that GAL receptors are expressed in rodent skin as well; however, the lack of specific GAL receptor antibodies for this species explains the deficit of data regarding the cellular distribution of GAL receptors on mouse and rat skin (Lu et al., 2005). As a vasoactive peptide, GAL is able to block plasma extravasation induced by different stimuli such as histamine, substance P, or antidiromic C fiber stimulation in rodent and pigeon skin (Jancso et al., 2000; Xu et al., 1991). Furthermore, after cutaneous treatment with tumor necrosis factor-α (TNF-α), heat, or a combination of substance P and calcitonin gene-related peptide, GAL-knockout (KO) mice lack neutrophil accumulation in the skin (Schmidhuber et al., 2008). In the K/B × N serum transfer model of autoimmune arthritis, an increase in clinical severity and vascular hyperpermeability in GAL3-R−knockout (GAL3-KO) animals compared with wild-type (WT) littermates was observed (Botz et al., 2016b), suggesting an anti-inflammatory role of GAL3-R. However, GAL3-KO mice showed no change in the progression of oxazolone-induced contact dermatitis compared with WT mice (Botz et al., 2016a). The absence of an obvious phenotype of GAL3-KO mice in the dermatitis model and the discrepancy between the arthritis and oxazolone models highlight the complexity of the role of GAL3-R in inflammation, which seems to depend on the type of inflammatory disease and the tissue affected.

Psoriasis is an inflammatory skin disease characterized by epidermal hyperplasia; micro-abscesses; neo-angiogenesis; a proinflammatory cytokine environment involving alterations of IL-17A, IL-23, and TNF-α; and activation of and infiltration by macrophages, mast cells, neutrophils, CD11c+ dendritic cells, and T cells (Elder et al., 2010; Ha et al., 2014). Topical application of imiquimod (IMQ), an innate toll like receptor 7/8 ligand, induces a dermatitis closely resembling human psoriasis. Similar to psoriasis observed in the clinic, inflamed, scaly, and plaque skin lesions; epidermal hyperproliferation; abnormal differentiation; and neo-vascularization occur upon IMQ treatment. This is caused by the infiltration of neutrophils and macrophages and the activation of mast cells, followed by recruitment of specific dendritic cells and T cells (Brembilla et al., 2017; Cai et al., 2011; Pantelyushin et al., 2012; Schon and Schon, 2007; van der Fits et al., 2009). There is clinical evidence that absence of neural input resulting from denervation injury leads to improvement of psoriasis, which suggests a role of the nervous system in the pathophysiology of this skin disease (Zhu et al., 2016). For example, α-melanocyte—stimulating hormone ameliorates inflammation in an IMQ-induced psoriasiform-inflammation model (Auriemma et al., 2012). Recently, a topical formulation of α-melanocyte—stimulating hormone was developed that reduced IMQ-induced psoriasiform inflammation in mice (Shah et al., 2016).

The aim of this study was to determine GAL receptor expression in human psoriatic skin and to elucidate progression of inflammation in murine models of psoriasis in GAL2-KO and GAL3-KO animals.

RESULTS

Co-expression of GAL3-R and CD31 in human vessels

Although GAL binding sites have been detected around dermal blood vessels (Kofler et al., 2004), evidence concerning which GAL receptor is expressed in the dermal vasculature is missing. Laser microdissection of human dermal blood vessels showed expression of GAL3-R but not GAL1-R and GAL2-R mRNA (see Supplementary Figure S1 online). To verify GAL3-R expression in the vasculature of human skin at the protein level and to determine whether GAL3-R expression differs in healthy versus psoriatic skin samples, we performed immunofluorescence staining of normal and psoriatic human skin samples. We used antibodies against GAL3-R, CD31, and α-smooth muscle actin to discriminate between CD31-positive endothelial cells (Figure 1a) and α-smooth muscle actin—positive pericytes (Figure 1b). GAL3-R staining (Figure 1c) was observed around large, mature vessels of male and female donors in CD31-positive endothelial cells, whereas the α-smooth muscle actin—positive pericytes remained GAL3-R—negative (Figure 1d).

In the skin we also detected GAL2-R— and GAL3-R—positive structures such as sweat gland cells and structures exclusively positive for GAL2-R such as epidermal keratinocytes, which is in line with the literature (Bovell et al., 2012; Dallos et al., 2006). A few GAL2-R— and GAL3-R—positive neutrophils and macrophages were observed in healthy skin. In addition, no GAL2-R expression was found in dermal blood vessels. An overview of GAL2-R and GAL3-R staining in different skin structures is depicted in Supplementary Figure S2 online.

GAL3-R overlaps with nestin staining in psoriatic microvessels

To analyze whether GAL3-R might also be present in proliferating, newly formed vessels, we stained skin biopsy samples of healthy and psoriatic individuals with anti-GAL3-R and nestin antibodies. Nestin serves as a marker for neural stem cells and angiogenesis, the latter a key pathogenic factor in psoriasis (Heidenreich et al., 2009; Schonthaler et al., 2009; Teranishi et al., 2007). In normal skin, GAL3-R (Figure 2a, 2c) but no nestin staining (Figure 2b, 2c) was observed in large, mature vessels. However, in newly formed microvessels, GAL3-R (Figure 2d) and nestin staining (Figure 2e) co-localized (Figure 2f), suggesting GAL3-R involvement in neo-angiogenesis.

The immunohistochemical staining of GAL2-R and GAL3-R of psoriatic skin shows similar expression patterns as nonpsoriatic skin. Immune cells such as neutrophils and macrophages were stained positive for both GAL2-R and GAL3-R (see Supplementary Figure S3 online), indicating that GAL receptor signaling on immune cells could contribute to psoriasis progression.

Altered response toward IMQ in GAL3-KO but not GAL2-KO mice

Because neo-angiogenesis plays an important role in psoriasis, and because GAL2-R and GAL3-R are expressed in skin
GAL3-R was shown to influence inflammation in different inflammatory diseases, we compared the progression of psoriasis in GAL2-KO and GAL3-KO versus WT control mice using the IMQ model (van der Fits et al., 2009).

Psoriasis clinical severity was evaluated by semi-quantitative scoring of erythema, scaling, and skin thickening. Erythema, which is an indicator of increased vascular permeability and inflammation, was significantly reduced in GAL3-KO compared with WT mice on day 5 \( (P = 0.0011) \) (Figure 2a), whereas GAL2-KO mice showed no differences in erythema development compared with WT mice (see Supplementary Figure S4a online). Psoriasis-typical scaling, which occurs due to hyperproliferation of keratinocytes, was also reduced in the GAL3-KO group on days 5 \( (P = 0.0015) \), 6 \( (P = 0.0001) \), and 7 \( (P = 0.0118) \) (Figure 2b) but not in GAL2-KO mice (see Supplementary Figure S4b). Thickening of the skin, which results from combined edema formation and immune cell infiltration as well as from acanthosis, was significantly lower on day 4 \( (P = 0.0020) \) in GAL3-KO mice (Figure 3c). This was the only parameter decreased in GAL3-KO mice (day 5, \( P = 0.0001 \)) (see Supplementary Figure S4c).

The cumulative score showed that GAL3-KO animals exhibited a reduced psoriatic response toward IMQ treatment compared with WT animals on days 4 \( (P = 0.0144) \), 5 \( (P = 0.0003) \), and 6 \( (P = 0.0341) \) (Figure 3d), as displayed in Figure 3g (representative images). GAL2-KO showed no differences in the overall cumulative disease severity score compared with WT mice at any time point (see Supplementary Figure S4d).

Epidermal hyperplasia produces scaling; therefore, we analyzed epidermal thickening in skin sections upon IMQ treatment. GAL3-KO animals showed a reduced thickness of the epidermis after IMQ treatment compared with WT mice on day 4 (66.3 \( \pm \) 8.8 \( \mu \)m vs. 53.5 \( \pm \) 12.3 \( \mu \)m, \( P < 0.01 \)) (Figure 3e, Supplementary Figure S5 online). In contrast, GAL2-KO animals displayed no differences in epidermal thickening compared with their corresponding WT mice on days 4 and 7 (see Supplementary Figure S4e). Upon psoriasiform inflammation, it has been reported that splenic mass increases upon IMQ treatment because of a systemic effect of the compound (Flutter and Nestle, 2013). GAL3-KO and WT mice displayed marked splenomegaly upon IMQ treatment, resulting in equal enlargement in WT mice (0.084 \( \pm \) 0.020 g to 0.154 \( \pm \) 0.018 g) and GAL3-KO mice (0.102 \( \pm \) 0.036 g to 0.156 \( \pm \) 0.022 g) on day 4. The splenic swelling progressed in both genotypes; however, we observed a smaller increase in spleen weight in GAL3-KO mice (0.217 \( \pm \) 0.042 g).
compared with WT mice (0.244 ± 0.045 g) on day 7 (P < 0.05) (Figure 3f). In GAL2-KO and their corresponding WT mice, IMQ-induced spleen enlargement was increased similarly (see Supplementary Figure S4f), indicating that although GAL2-R is expressed in the mouse skin, only GAL3-R seems to influence the progression of psoriasis.

**GAL3-KO mice show delayed neovascularization**

Angiogenesis is a key pathogenic factor in psoriatic disease progression (Heidenreich et al., 2009). Thus, we quantified CD31-positive vessels in tissue sections of control and IMQ-treated dorsal mouse skin. We observed an increase in vessel density in WT animals 4 and 7 days after IMQ treatment compared with vehicle-treated control skin (P < 0.001). GAL3-KO animals showed no IMQ-induced up-regulation of vessel number on day 4. However, on day 7, the vessel density was increased in IMQ-treated GAL3-KO mice compared with untreated GAL3-KO mice (P < 0.001). The difference in number of vessels between IMQ-treated GAL3-KO mice and WT mice on day 4 was significant (P < 0.01).
however, the difference was diminished on day 7 (Figure 4a). No differences in vessel densities between GAL2-KO and WT mice upon vehicle or IMQ treatment were observed on days 4 and 7 (see Supplementary Figure S6 online). This finding is in line with the absence of a macroscopic difference between GAL2-KO and WT animals upon IMQ treatment.

To investigate whether GAL3-R deficiency influences angiogenesis directly or via regulation of the expression of vascularization factors, we performed an expression analysis of vascularization-related genes. The mRNA levels of vascular endothelial growth factor, angiopoietin I, and angiopoietin II were not different between genotypes in IMQ-treated skin on days 4 or 7 (Figure 4b–d). Because the amount of gene products related to vascularization is not different, this could be an indication of a direct involvement of GAL3-R signaling in neovascularization. This is strengthened by the finding that GAL3-R mRNA was present in vessels of healthy and inflamed skin (day 4 after IMQ treatment) (see Supplementary Figure S7 online).

GAL3-KO mice show reduced myeloperoxidase activity and reduced neutrophil infiltration

Because we observed differences in vessel densities and because angiogenesis is necessary for the sustained survival and accumulation of inflammatory cells under chronic inflammatory conditions (Prescott et al., 1984), we hypothesized that neutrophil influx might be affected in psoriatic GAL3-KO animals. Therefore, we quantified the amount of neutrophil-related myeloperoxidase (MPO) in control and IMQ-treated skin biopsy samples. We detected an increase on day 4 followed by a return to control levels on day 7 in both WT and GAL3-KO animals. On day 4 the amount of MPO was lower in the GAL3-KO compared with the WT group (0.098 ± 0.047 U/ml vs. 0.143 ± 0.055 U/ml, respectively; P < 0.01) (Figure 5a).

To determine if the lower amount of MPO was a consequence of different amounts or subtypes of neutrophils or neutrophil-attracting macrophages, we quantified NIMP-R14⁺ neutrophils and F4/80-positive macrophages in the skin sections. No differences in macrophage abundance (Figure 5b) or expression of macrophage M1 and M2 subtype-related genes (Jablonski et al., 2015) (i.e., Arg1, Il12b, Fpr2, and Egr2 [see Supplementary Figure S8 online]) were observed in the skin sections of the two different genotypes of untreated and IMQ-treated mice on day 4 and day 7. However, a trend toward reduced NIMP-R14⁺ neutrophils was observed in the skin sections of GAL3-KO compared with WT mice upon IMQ treatment on day 4 (P = 0.08). In vehicle-treated skin no quantifiable amount of neutrophils was detected (Figure 5c). In line with the lack of a macroscopic
phenotype, GAL2-KO and corresponding WT animals showed no difference in the number of NIMP-R14+ neutrophils on day 4 of IMQ treatment (see Supplementary Figure S9a online). Because neutrophils are key effector cells in IMQ-induced psoriasis, the reduced MPO amount, together with the trend toward reduced numbers of neutrophils in the GAL3-KO mice, supports the observed reduced psoriasis severity score in this transgenic mouse strain.

Genotype-specific modulation of cytokine expression

We next examined if the reduced severity of the IMQ-induced psoriasis in GAL3-KO animals could be due to alterations in the expression of psoriasis-related cytokines. We detected lower expression of IL-17A (65% reduction, \( P < 0.001 \)), IL-22 (80% reduction, \( P < 0.001 \)), IL-23 (64% reduction, \( P < 0.01 \)), and TNF-\( \alpha \) (49% reduction, \( P < 0.05 \)) mRNA in GAL3-KO compared with WT animals on day 4 (Figure 6a). On day 7, after the climax of inflammation, a decline in cytokine mRNA levels was noticed, and the significant differences between the genotypes disappeared (Figure 6b). In GAL2-KO animals, no differences in the mRNA expressions of IL-17A, IL-22, IL-23, and TNF-\( \alpha \) on day 4 (see Supplementary Figure S10a online) or day 7 were detected compared with the WT (see Supplementary Figure S10b). The PCR primers and reaction conditions are provided in Supplementary Tables S1 and S2 online. To confirm that the reduced amount of IL-17 and IL-22 observed in the GAL3-KO animals is originating from a reduced number of IL-17- and IL-22-producing neutrophils and not mast cells (Mashiko et al., 2015), we determined the amount of toluidine blue-stained mast cells in the skin sections. No differences in mast cell numbers in control and IMQ-treated skin sections of GAL3-KO and WT animals on day 4 were detected (Figure 5d). As expected between GAL2-KO and their corresponding WT animals, the amount of mast cells did not differ (see Supplementary Figure S9b).

Consequently, the amount of mast cells does not account for the altered expression of IL-17A and IL-22. Possibly, neutrophils that are also capable of producing these interleukins (Taylor et al., 2014) are responsible for the alterations in cytokine levels.

DISCUSSION

In this study, we showed that endothelial cells of the dermal vasculature of healthy individuals express GAL3-R and that nestin-positive neovessels in psoriatic skin co-express GAL3-R. Nestin, a type VI intermediate filament protein, is a selective marker for bone marrow-derived mesenchymal stem cells and is expressed on perivascular and endothelial cells during angiogenesis in different tissues (Suzuki et al., 2010; Xie et al., 2015). GAL2-R and GAL3-R mRNA is expressed in mouse embryonic cells, and GAL expression was observed during embryonic development (Jonas et al., 2009; Tarasov et al., 2002). These data indicate a potential role of GAL3-R in stem cell function and suggest that it may have a neo-angiogenic effect.

A recent study reported the distribution of GAL receptors in the human eye, which is a model organ for studying vascularization: all three GAL receptor types were found to be expressed on choroidal blood vessels, GAL1-R and GAL3-R on iris vessels, and GAL3-R alone on ciliary vessels, indicating localization and tissue-specific expression patterns of GAL receptors (Schrodl et al., 2015). The abundance of GAL receptors in the eye could indicate a role in vascularization-related events.
In psoriasis development, a small population of mast cells and macrophages residing in the skin can act as sentinel cells that initiate neutrophil recruitment by increasing the permeability of local blood vessels and the release of chemokines (Ajuebor et al., 1999; Vieira et al., 2009). The first wave of infiltrating immune cells after IMQ treatment consists of neutrophils. On days 2 to 4 a second wave of neutrophils is accompanied by macrophages (Flutter and Nestle, 2013). We observed an increase in macrophages and neutrophils after 4 days of IMQ treatment and a reduction of both cell types by day 7. Molecular analysis of the skin showed no abnormalities in M1- and M2-related genes between WT and GAL3-KO animals. In skin sections of GAL3-KO animals, a trend toward a lower amount of Fpr2 expression was observed on day 4. Because Fpr2 is also present on neutrophils (Hartt et al., 1999) and plays a role in homing of endothelial progenitor cells (Heo et al., 2014), which show stemness characteristics and are crucially involved in the formation of vascular networks, we hypothesize that the lower Fpr2 levels in GAL3-KO animals may contribute to the vascular phenotype in vivo.

Neovascularization upon IMQ treatment was observed to different degrees in WT and GAL3-KO mice, whereas the increase in vascular endothelial growth factor mRNA levels was genotype independent. Because we observed lower levels of the cytokines IL-17A, IL-22, IL-23, and TNF-α in IMQ-treated skin of GAL3-KO mice, we addressed the question of whether the lower cytokine levels could be linked to IL-17— and IL-22—expressing mast cells or neutrophils (Mashiko et al., 2015; Taylor et al., 2014). Because mast cells in the skin originate from progenitors but neutrophils are recruited from blood into the skin, altered neovascularization occurring during the course of disease could contribute to variations in neutrophil number between WT and GAL3-KO mice. Indeed, we observed lower numbers of neutrophils but not mast cells in GAL3-KO compared with WT mice, which is in line with the reduced MPO activity. Furthermore, this supports our hypothesis that the expression of GAL3-R on blood vessels is important for disease progression. Accordingly, we detected GAL3-R mRNA in human and murine dermal vessels.

Recent advances in the GAL field led to the development of specific peptidergic agonists and antagonists with higher specificity and increased stability (Lang et al., 2015). Despite the major effort of several research groups to generate non-peptidergic ligands (Bulaj et al., 2008; Reyes-Alcaraz et al., 2016), the only available non-peptidergic GAL3-R ligands are SNAP 37889 and SNAP 398299, a less soluble derivative of SNAP 37889 (Swanson et al., 2005). In the skin it was shown that SNAP 37889 blocks plasma extravasation induced by substance P and calcitonin gene-related peptide injection (Schmidhuber et al., 2009). Further research with this antagonist showed unspecific toxic effects on immune cells when used in high concentrations (Koller et al., 2016). Therefore, local administration, especially on the skin, could potentially be toxic to immune cells, and thereby mimic an anti-inflammatory effect. However, intraperitoneal administration of SNAP 37889 has been reported to have anti-inflammatory effects in a model of murine pancreatitis and to suppress alcohol drinking and morphine self-administration in mice (Barreto et al., 2011; Scheller et al., 2017). In these studies no adverse effects were reported, possibly because of the intraperitoneal route of administration.
administration. Thus, further studies should investigate if a dermal application of SNAP 37889 is feasible and could be used to treat psoriasis.

Future studies should investigate the direct effects of the GAL system on endothelial cells because they represent a link between nerve-derived neuropeptides and immune cells. Furthermore, studies should aim at developing stable, nontoxic GAL analogs for the treatment of psoriasis and other inflammatory diseases.

**MATERIALS AND METHODS**

**Human tissue samples**

Experiments were performed in accordance with the Helsinki Declaration of 1975 (revised in 1983) and the guidelines of the Salzburg State Ethics Research Committee, because this was neither a clinical drug trial nor an epidemiological investigation. Upon hospital admission, all patients (white, male and female, age range = 25–62 years) signed informed consent forms concerning the surgical removal of the skin biopsy samples. The study did not extend to examination of individual case records. The anonymity of the patients has been ensured.

**Psoriasis in vivo model**

Male and female mice 8–10 weeks old received a daily topical dose of 62.5 mg of the commercially available IMQ cream (5%) (Aldara; 3M Pharmaceuticals, Neuss, Germany) applied to a shaved and depilated back for 3 or 6 consecutive days, translating to a daily dose of 3.125 mg of the active compound, as published previously (van der Fits et al., 2009). Control mice were treated similarly with a control vehicle cream (Vaseline Lanette cream, Fagron, Barsbüttel, Germany).

Additional information on experimental animals, the psoriasis in vivo model, evaluation of skin inflammation (van der Fits et al., 2009), MPO assay (Schiervagen et al., 1990), RNA isolation, expression analysis, immunohistochemistry, data analysis, and statistics are provided in the Supplementary Materials online.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.


