Molecular mechanisms of pruritus in cholestasis
Kremer, Andreas

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SUMMARIZING DISCUSSION

Modified from

Beuers U, Kremer AE, Bolier R, Oude Elferink RP

SUMMARIZING DISCUSSION

The observation that pruritus accompanies jaundice has already been made by the ancient Greek physician Aretæus the Cappadocian in the 1st century AD.\(^1\) Today chronic pruritus - which is defined by duration of more than six weeks - is well recognized as a frequent and agonizing symptom accompanying many types of liver diseases, particularly those with cholestatic features.\(^2\)\(^-\)\(^4\) Beside fatigue, chronic pruritus represents a major burden of patients with hepatobiliary disorders and can dramatically reduce quality of life.\(^5\) In these patients itching may be mild and tolerable, but does in some patients limit daily life activities, cause severe sleep deprivation resulting in lassitude, fatigue, depressed mood and even suicidal sensation. In rare cases, intractable pruritus has become a primary indication for liver transplantation.\(^4\)\(^,\)\(^6\)\(^-\)\(^8\)

Pruritus of cholestasis is characterized by a circadian rhythm with patients reporting the highest intensity in the evening and early at night,\(^9\) but it should be mentioned that chronic pruritus in general tends to increase with warmth and at night. A predilection site of pruritus in cholestatic patients are the limbs and in particular the palms and soles,\(^10\)\(^,\)\(^11\) albeit pruritus may be generalized in many patients. Patients may report that scratching barely alleviates itch sensations and that pruritus is accompanied with other sensations such as stinging and burning. Furthermore, female cholestatic patients commonly report pruritus worsening during progesterone phase of the menstrual cycle, in late pregnancy, and during hormone replacement therapy.\(^9\)\(^,\)\(^12\) In multivariate analysis, serum alkaline phosphatase and the Mayo risk score were found to be independent indicators for the occurrence of pruritus in 335 PBC patients.\(^13\) In contrast to pruritus in dermatological disorders, primary skin lesions are not detectable in cholestatic patients, however intense scratching activity may cause secondary skin alterations such as excoriations and prurigo nodularis.\(^14\) Although secondary skin lesions may be difficult to discriminate from primary skin disorders, if no scratch tools are used the so-called “butterfly sign” points to a non-dermatological cause of chronic pruritus. This sign is defined as unaffected skin at the upper patient’s back due to difficulties to manually reach that part of the body. Furthermore, typical skin signs of chronic liver disorders such as jaundice, spider naevi, palmar erythema or leuconychia may help to identify the underlying cause.
The search for the itch-causing substances in cholestasis has been ongoing for 2000 years when Aretæus the Cappadocian stated that “pruritus in jaundiced patients is caused by prickling biliary particles”.\(^1\) Our state of knowledge at the start of the 21\(^{\text{st}}\) century was not much different. Still, clinical observations have led to the conclusions that potential pruritogens (i) accumulate in the systemic circulation as indicated by (partial) relief of severe pruritus after treatment with plasmapheresis, albumin dialysis (e.g. MARS\(^{\text{\textregistered}}\), Prometheus\(^{\text{\textregistered}}\)) or plasma separation/anion absorption; (ii) are secreted into bile as suggested by attenuation of pruritus after oral administration of the anion exchange resin cholestyramine or proven by rapid relief of most severe, treatment-refractory pruritus after nasobiliary drainage; (iii) are (biotrans-)formed in liver and/or gut as indicated by effective treatment with the potent pregnane X receptor (PXR) agonist, rifampicin; and (iv) affect the endogenous opioidergic and serotonergic system as suggested by mild antipruritic activity of opioid antagonists such as naltrexone and serotonin reuptake inhibitors such as sertraline.\(^2,15,16\) In the past various substances among which are histamine, serotonin, bile salts, endogenous opioids, and progesterone metabolites have been proposed as pruritogens but with limited evidence. It is therefore likely that these substances do not represent the direct neuron activating molecules, but they may modulate or sensitize sensory neurons thereby contributing to itch sensation. With this thesis we added lysophosphatidic acid (LPA) as novel potential pruritogen in cholestasis. These novel findings have renewed our view on the pathogenesis of pruritus and may open new avenues for anti-pruritic treatment strategies. The role of the ATX-LPA-axis in cholestatic pruritus, the recently described G protein-coupled receptor TGR5 on sensory neurons and the novel insights into the neuronal signalling pathways of pruritus are discussed below in detail.

**Bile salts as pruritogens**

During cholestasis many cholephiles among which are bile salts (and their protonated form, bile acids), bilirubin and steroid metabolites accumulate in circulation and tissues. The removal of bile from the body either by external biliary diversion\(^17-19\) or nasobiliary drainage\(^20-22\) quickly and dramatically alleviates severe cholestatic pruritus. Thus, certain biliary substances contribute either directly or indirectly to the onset of itching. The notion
that bile salts represent the itch-causing agents was further supported by the observations that feeding of bile salts to cholestatic patients aggravated their pruritus, intradermal injection of bile salts, albeit at supra-pathophysiological concentrations, caused pruritus in healthy volunteers and anion exchange resins, which bind bile salts inside the intestinal lumen, ameliorate pruritus.

Bile salts mediate their effects via the nuclear transcription factor farnesoid X receptor (FXR) or the transmembrane G protein-coupled receptor TGR5. Upon binding to these receptors bile salts are capable of activating complex transcriptional networks and intracellular signaling cascades. Activation of FXR has been proven to exert various beneficial effects in different pathophysiological states including cholestasis, liver fibrosis, non-alcoholic steatohepatitis and hepatocellular carcinoma. The semi-synthetic bile salt obeticholate (= 6-ethyl-chenodeoxycholate) is a selective FXR ligand which is currently studied in clinical trials in patients with primary biliary cirrhosis (PBC) and NASH. This drug exerted beneficial anti-cholestatic effects in PBC, however, caused pruritus particularly at high doses. The observation that PBC patients receiving the highest dose of 50 mg in the phase II trial had a significant increase in serum ATX activity may only explain this phenomenon in part. In contrast to humans, feeding OCA to wild-type or cholestatic mice such as Mdr2−/− on cholate diet did not result in increased scratching activity (unpublished data), indicating that FXR-mediated itch sensation may be limited to certain species. The underlying mechanism of FXR agonists induced pruritus remains to be elucidated.

Notably, Alemi and colleagues recently postulated that the bile salt receptor TGR5 may play a role in the generation of cholestatic itch. They showed that TGR5 is expressed on neurons in the dorsal root ganglia but not in the skin of mice and this expression partially overlaps with TRPV1 and GRP. They also demonstrated bile salt-induced activation of mouse DRG neurons. Oleanolic acid, a TGR5 agonist was also capable of activating these cells. Importantly, in vivo experiments in TGR5 overexpressing mice revealed an increased basal scratch activity. Intradermal injection of 25 μg deoxycholate (DCA) evoked scratch behaviour in normal mice which was partly reduced in TGR5−/− mice and increased in the overexpressing mice. The same group further showed that, in HEK cells overexpressing TGR5 and TRPA1 as well as in primary neurons coexpressing these
two receptors, bile salts induced activation of TRPA1 which depended on the presence of TGR5. In vivo DCA-induced itching was reduced by antagonists of TRPA1 and in TRPA1−/− mice. These findings would support a primary role for TGR5 in itch induction. Unfortunately, for most of the in vitro experiments relatively high concentrations of unconjugated DCA (up to 100 μM) were used. These concentrations are much higher than those observed in pathological conditions like PBC or ICP which are associated with pruritus. In contrast, PBC and ICP are characterized by often only moderate elevation of serum bile salt concentrations and typically a depleted DCA pool size. In particular unconjugated bile salts such as DCA barely exist in serum and bile in vivo. Also in the in vivo experiments a relatively high dose of 25 μg DCA was used, which may lead to activation and degranulation of mast cells. Furthermore, even with the most sophisticated technique no correlation between the concentration of any naturally occurring bile salt in the circulation, urine or skin and severity of pruritus could be proven – also shown for serum in chapter 3 of this thesis. Many patients, particularly those with obstructive cholestasis, have highly elevated levels of serum bile salts but never experience pruritus. In contrast, women with intrahepatic cholestasis of pregnancy presenting with the mildest form of cholestasis with only marginally increased bile salt concentrations do by definition suffer from pruritus. In addition, the enzyme inducers phenobarbital and rifampicin effectively improved pruritus without changing or with only temporarily decreasing the levels of serum bile salts. However, these considerations should not minimize the importance of the study of Alemi et al. as they do not exclude the possibility that, besides other factors, TGR5 activation may contribute to itch perception. It is well documented that neurosteroids more than bile salts may activate TGR5 in the central nervous system. Notably, progesterone has recently been shown to activate TGR5 in placental tissue in a dose-dependent manner. Of note, progesterone metabolites such as 5β-pregnan-3α,20α-diol-3-sulfate have been increased in women with intrahepatic cholestasis and dropped upon UDCA treatment. More importantly, these molecules were capable of activating TGR5 in vitro and inducing scratching behavior in vivo which was attenuated in TGR5−/− mice (Abu-Hayyeh S et al, submitted). In summary, rather the type of hepatobiliary disease and not simply the level of bile salts or the magnitude of cholestasis may determine itching in cholestasis.
Chapter 8

Lysophosphatidic acid as pruritogen

Beside bile salts, a variety of molecules such as endogenous opioids, histamine, serotonin, and steroid metabolites have been discussed as potential pruritogens in cholestasis.\(^9,16\) Serum and/or tissue levels of these candidates have not been shown to correlate with itch intensity making them less likely to be responsible for pruritus of cholestasis. Endogenous opioids or serotonin might rather modulate than initiate the complex signalling cascades leading to the desire to scratch. Thus, it was the aim of this thesis to identify the causal pruritogens in serum samples of cholestatic patients suffering from pruritus. In **chapter 3** we were able to identify the small phospholipid lysophosphatidic acid (LPA) as potential candidate for the initiation of itch in cholestasis. We had hypothesized that if a pruritic factor exists in serum of patients with pruritus due to cholestasis this factor should potentially activate neuronal cells. We therefore exposed various neuronal cell lines to sera of pruritic and non-pruritic cholestatic patients as well as healthy volunteers and compared the cytosolic free \(\text{Ca}^{2+}\) [\(\text{Ca}^{2+}\)] response as a simple marker of cell activation. We detected a particularly strong [\(\text{Ca}^{2+}\)] response induced by sera of women with intrahepatic cholestasis of pregnancy (ICP).\(^50\) Chemical analysis revealed that the neuronal activator in these sera was not a peptide, had a molecular weight below 3 kDa, was amphiphilic with increasing hydrophobicity upon protonation, and acted via G-protein coupled receptors. Our hypothesis that the molecule was lysophosphatidic acid (LPA) was verified by repression of the serum-induced neuronal activation by a LPA receptor blocker, and demonstration of elevated levels of LPA in sera of women with ICP by HPLC-MS/MS. In addition, a causative role for LPA was suggested by the finding that intradermal injection of LPA but not vehicle initiated a scratch response in mice, confirming a previous independent study in mice.\(^50,51\) Thus, we were able to identify a potent neuronal activator in serum which represents a pruritogen upon intradermal injection in mice. These data are strengthened by the observation that LPA is also capable of inducing itch sensation in healthy human volunteers upon intradermal application via heat-inactivated cowhage spiculae (unpublished data).
Autotaxin activity correlates with itch intensity

Of note, LPA levels were rather unstable in patient serum and increased in concentration upon storage. Therefore, we determined the activity of the lysophospholipase D, autotaxin (ATX), which is responsible for formation of LPA from its precursor lysophosphatidylcholine.\textsuperscript{52,53} ATX is the main source of circulating LPA levels.\textsuperscript{54} ATX belongs to the family of ectonucleotide pyrophosphatases / phosphodiesterases (ENPP1–7) and is also referred to as ENPP2. This large glycoprotein is synthesized as a pre-proenzyme and, in contrast to all other ENPPs, secreted to the extracellular space upon two N-terminal cleavages.\textsuperscript{55} As a pyrophosphatase ATX hydrolyses nucleotides, but with a much lower affinity compared to lysophospholipids.\textsuperscript{56} The specificity for lysophosphatidylcholine (LPC) as a substrate is probably further increased by the fact that formed LPA remains in the binding pocket, thereby inhibiting the enzyme. From this position LPA can only be displaced by LPC and not by nucleotides. The crystal structure of ATX was recently resolved and these data show that the enzyme has a hydrophobic pocket that allows binding of the substrate lysophosphatidylcholine. Two N-terminal somatomedin B–like domains mediate binding of ATX to plasma membrane integrins.\textsuperscript{57} These observations led to the view that ATX binds to the plasma membrane of target cells via integrin β1 or 3 and locally generates LPA which can subsequently bind to its cognate receptors.\textsuperscript{55} ATX has been implicated in many (patho)biological processes including cell survival, proliferation, differentiation, and migration. ATX is involved in vascular and neural development, wound healing and neuropathic pain, but also cancer development.\textsuperscript{56,58} In this thesis we add a role for ATX in the pathogenesis of pruritus.

In chapter 3, 4 and 6 we could show that serum ATX activity and ATX protein levels were markedly higher in adult and pediatric cholestatic patients with pruritus than in cholestatic patients without pruritus which were in turn higher than in healthy volunteers.\textsuperscript{50} In chapter 5 we add that serum activity of autotaxin is increased in ICP compared to other pruritic disorders of pregnancy, pre-eclampsia complicated by HELLP-syndrome, and pregnant controls. Autotaxin had an excellent sensitivity and specificity in diagnosing ICP from other pruritic disorders or pre-eclampsia/HELLP-syndrome and could be useful as a diagnostic marker. Furthermore, in chapter 4 serum ATX activity in cholestatic patients was shown to be also higher than in patients with pruritus due to chronic kidney disease,
Hodgkin’s disease or atopic dermatitis suggesting that strong serum ATX elevation is rather specific for cholestatic itch.\textsuperscript{59} It is important to note, however, that patients with Hodgkin’s disease as well as patients with atopic dermatitis do have significantly elevated ATX levels in blood compared to healthy controls, although this could not be correlated to the extent of itch in our patient cohorts.\textsuperscript{59} Recently, others similarly found increased levels of ATX in serum of atopic dermatitis and even described a correlation with itch intensity.\textsuperscript{60,61} A role of ATX in other forms of pruritus, therefore, cannot be excluded. In these conditions local ATX production could be more important than systemic levels. Further studies on ATX expression in skin biopsies or tumor tissue would be required to further correlate local levels with presence of itch perception. In addition, local LPA concentrations in the skin, e.g. attained by skin dialysis, may shed light on the role of the ATX-LPA-axis in itch sensation in these patients.

Notably, in chapter 3 we could show that in cholestasis, serum ATX activity, but not other putative markers of itch such as histamine, serum bile salt levels or serum \(\mu\) opioid activity were correlated with itch intensity. Furthermore, in chapter 4 it is shown that ATX serum activity mirrored treatment response to therapeutic interventions such as the anion exchange resin, colesevelam, the potent pregnane X receptor (PXR) agonist rifampicin, nasobiliary drainage, or MARS treatment.\textsuperscript{59} Rifampicin was found to reduce ATX expression at the transcriptional level in human liver derived cell lines by a PXR-dependent mechanism, possibly explaining the strong antipruritic effect of rifampicin in clinical practice at least in part.\textsuperscript{59} Thus, the ATX-LPA-axis is likely to represent a key element in pruritus of cholestasis but may also play a role in other forms of chronic pruritus such as atopic dermatitis.

\textbf{Source of autotaxin in cholestatic liver disease}

Although the identification of the ATX-LPA-axis as a key factor in cholestatic pruritus represents a real opportunity for development of causal therapy, several questions remain to be answered. The source of circulating levels of ATX has not been identified so far. ATX expression on mRNA levels could be determined in various tissues and organs such as brain, adipose tissue, lung, liver, intestine, kidney, ovary and high endothelial venules.\textsuperscript{62} Which of these organs contribute to the circulating ATX levels being present in human
Summarizing discussion

plasma and cerebrospinal fluid has so far not been established. Preliminary evidence suggests that the human small intestine may be a major contributor (Bolier et al., in preparation) and that a yet to be identified factor “X” in the enterohepatic circulation may drive ATX expression in cholestasis (s. Figure 1).

In mice, radioactive-labelled ATX was shown to be largely cleared from the circulation by the liver with a half-life time of a few minutes. The authors suggested that ATX was cleared from plasma by scavenger receptors on liver sinusoidal endothelial cells. Thus, beside increased expression reduced clearance of circulating ATX could also be responsible for the increased ATX levels in cholestasis. Preliminary data from our group, however, show that clearance of recombinant ATX was comparable in cholestatic and non-cholestatic ATP8b1 knock-out mice (unpublished data).

Upon interruption of the enterohepatic circulation by nasobiliary drainage, circulating levels of ATX rapidly dropped concomitant with relief of pruritus as shown in Figure 1: Model of development and influence by therapeutic interventions of cholestatic pruritus.63

<table>
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<tr>
<th>LPA</th>
<th>Autotaxin</th>
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<tr>
<td>Sertraline</td>
<td>Naltrexone</td>
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<td>Itch neuron</td>
<td>Albumin dialysis, plasmapheresis</td>
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<td>Rifampicin</td>
<td>Nasobiliary drainage</td>
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<td>LPA</td>
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<td>Cholestyramine</td>
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<td>Small intestinal cell</td>
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<td>Nasobiliary drainage</td>
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<td>Factor “X”</td>
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Rifampicin
However, ATX is not secreted into bile (chapter 3), indicating that a factor within the enterohepatic circulation is responsible for the increased ATX levels. Steroid hormones may be one of the classes of compounds that cause induction of ATX expression. Takeo et al. have shown that autotaxin (Enpp2) expression is upregulated in the hippocampus of ovariectomized rats upon treatment with estrogen. This observation is supported by chapter 5 in which we present data that healthy female individuals have significantly increased serum ATX levels when using oral contraceptives. Hence, induction of ATX by female steroid hormones appears relevant and may particularly play a role in pregnancy. On the other had it could also explain the higher frequency of itch in cholestatic female vs. male patients.

ATX expression is augmented upon treatment with cytokines such as TNF or IL-6 or growth factors such as EGF and bFGF, whereas expression is reduced by IFNγ, IL-1 and IL-4. It is possible that certain cytokines contribute to increased serum ATX levels during cholestasis, although in other states of inflammatory cytokine release pruritus is not a typical symptom. In chapter 6 we analyzed the effect of bile salts on ATX expression in vitro. FXR agonists, however, did not induce ATX expression. At present, it is unclear which compound(s) in the enterohepatic circulation may be responsible for direct or indirect induction of ATX expression.

ATX elevation can also occur in a number of non-cholestatic physiological states such as regular pregnancy and pathophysiological conditions including various cancer entities in which pruritus is not a clinical feature. Thus, other cholestatic factors may also play a role in initiation and/or potentiation of pruritus of cholestasis. These substances may bind to certain GPCRs such as the recently described mas-gene related G-protein coupled receptors (Mrg) on sensory neurons which are involved in itch signalling. Further screening on molecules binding to such itch-selective receptors will help to elucidate these co-factors.

**Neuronal signaling by LPA**

In chapter 3 a causative role for LPA-induced pruritus was suggested by the finding that intradermal injection of LPA caused a dose-dependent scratching behavior in mice, confirming a previous independent study in mice. A recent study confirmed LPA-induced scratching behavior which was unaffected by the absence of TGR5 in TGR5−/−
mice. Still, an unsolved issue is the precise mechanism of activation of itch-selective neurons by LPA. At present six G-protein coupled receptors for LPA (LPA1-6) have been well defined. These receptors are found on various tissues. LPA receptors are also present on peripheral sensory neurons that mediate various sensations to the central nervous system. LPA has been capable of inducing neuropathic pain via LPA1-, LPA3- and LPA5-receptors. Which LPA-receptor and intracellular signaling pathway are required for LPA-induced pruritus warrants further studies using knock-out animals. Of note, it was recently suggested that LPA may also directly activate the ion channel TRPV1. Nieto-Posadas and colleagues showed in patch clamp studies that LPA stimulates TRPV1 channel opening. This study suggests that LPA interacts with a proposed intracellular PIP2 binding site on TRPV1 in which the lysine 710 residue participates. It must be stressed therefore that these studies mainly address a role for intracellular rather than extracellular LPA. Indeed, activation of inside-out membrane patches (i.e. intracellular signaling) was considerably faster and stronger than in right side out patches (i.e. extracellular). Since LPA is a relatively hydrophilic phospholipid it remains to be shown how relevant this type of activation is with regard to extracellular generation of LPA. In fact, we were unable to show that extracellular LPA could activate heterologously expressed human TRPV1 in HEK cells. Furthermore, extracellular LPA activated a small percentage of DRG neurons which did not respond to capsaicin, indicating that these responsive neurons did not express TRPV1. Finally, using neurons of TRPV1 knock-out animals LPA could still activate a comparable subset of neurons (Kremer et al., in preparation). These contradictory results may be explained by different mode of action of intracellular and extracellular LPA. Which receptor and intracellular signalling pathway are required for LPA-induced pruritus warrants further investigation.

**Neuronal transmission of pruritus**

From an evolutionary perspective acute pruritus serves as an alarm signal to protect the body against potentially harmful environmental threats such as parasites, noxious plants or other irritants. The scratch response helps to remove these harmful agents from the skin and diminishes itch sensation. Acute pruritus is commonly associated with a reddish swelling of the skin induced after histamine release, e.g. from mast cells. The observed wheal and flare
reaction are mainly mediated by secondary release of vasoactive substances such as calcitonin gene-related peptide (CGRP) and substance P from axon collaterals. Antagonists of the H₁-receptor effectively alleviate these short-lasting acute forms of pruritus. These acute forms of pruritus are mainly mediated by histamine-responsive sensory neurons in the skin that are insensitive to nociceptive stimuli such as mechanical pain.

In contrast, chronic pruritus which per definition lasts longer than six weeks can be a seriously debilitating symptom accompanying various cutaneous and systemic disorders, but may also be caused by drugs such as the anti-malaria drug chloroquine or the volume expander hydroxyethyl starch. As antihistamines do not improve itching in most of these conditions, it is likely that itch sensation is mediated via histamine-independent pathways as outlined below.

Itch sensation depends on a complex interplay of pruritogens, their receptors on peripheral sensory nerve fibres, intraspinal and cerebral neural pathways, as well as cerebral processing of the stimuli. Almost a century ago, itch was regarded as a mild form of pain induced by weak activation of nociceptive nerve fibers. However, substances such as histamine and the spicules of the tropical fruit mucuna pruriens, cowhage, induce itch even at high concentrations whereas weak pain stimuli often do not induce itch. In 1997, Schmelz and colleagues provided evidence for primary afferent neurons specific for histamine in cutis and subcutis of human beings. These itch-specific unmyelinated C-fibers are insensitive to mechanically induced pain stimuli and transmit their signals from the skin through the dorsal root ganglia to a second neuron in lamina I of the dorsal horn of the spinal cord. These observations indicated that itch and pain are transmitted via itch-selective and pain-selective neurons, respectively. Interestingly, itch signals induced by histamine and cowhage are transmitted by mutually exclusive populations of neurons in the spinal cord supporting existence of different classes of pruritoceptive nerve fibres, as also known for nociceptive neurons. It was postulated that itch sensation evoked by a given stimulus modality would depend on a specific neuronal pathway, the itch-specific ‘labelled-line’. However, this theory is contradicted by the observation that these itch-selective neurons can also be activated by the algogen and TRPV1 agonist capsaicin. An elegant
animal study indicated that a small subpopulation of sensory neurons expressing the Mas-related G protein-coupled receptors subtype A3 (MrgA3) could represent itch-selective neurons.\textsuperscript{79} Scratching behaviour in mice was strongly attenuated to most intradermally applied pruritogens after ablation of these MrgA3 positive neurons. In a second step, mice lacking the TRPV1 channel were used and TRPV1 was re-expressed only in MrgA3 positive neurons. In these mice the algogen capsaicin largely caused scratching behaviour but hardly any pain-related wiping.\textsuperscript{79} Thus, irrespective of the modality of activation these neurons seem to induce itch sensation but no pain. Still, the itchy spicules of cowhage activated every single polymodal nociceptor tested in human microneurography.\textsuperscript{80} The cowhage enigma remains and seems to be incompatible with the labelled-line hypothesis, unless the highly focal stimulus of these spicules is taken into account. The spatial contrast theory may explain this paradox by stating that itch arises from a sharp contrast between individual nociceptors that are firing and their surrounding neighbour remain silent. Pain sensation occurs if the overlapping receptive fields are more homogenously activated.\textsuperscript{81}

Although there might be subsets of neurons selectively mediating itch and pain signals both pathways are closely intertwined processes: activation of pain neurons abrogates itch sensation, e.g. by scratching, cooling or heating of the skin.\textsuperscript{82,83} Analgesics may induce itch sensation, e.g. by epidural or intrathecal application of opioids or anaesthetics.\textsuperscript{84-86} These phenomena may be explained by an itch circuitry which is under a tonic inhibitory control of mechano-sensitive neurons (see Figure 5, chapter 2). Evidence for such an inhibitory control was supported by the observation of spontaneous intense scratching behaviour in mice lacking certain inhibitory, Bhlbh5- and Prdm8-expressing interneurons.\textsuperscript{87,88} These interneurons are believed to be activated by glutamate. Interestingly, deletion of the glutamate transporter VGLUT2 also strongly augmented spontaneous and induced scratching behaviours after application of pruritogens which underlined this hypothesis.\textsuperscript{89,90}

Several receptors have been implicated in the onset of itch sensation.\textsuperscript{91} The group of mas-related G protein-coupled receptor (Mrgs) represents a family of GPCRs consisting of more than 50 members in the mouse genome. Several of these Mrgs are specifically expressed in small-diameter sensory neurons in dorsal root and trigeminal ganglia,
indicating their important role in somatosensation. Recently, several of Mrgs have been identified as receptors that mediate non-histaminergic itch: the anti-malaria drug chloroquine was shown to activate Mas-related G protein-coupled receptor subtype A3 (MrgA3). The bovine adrenal medulla 8–22 peptide (BAM8-22), which induces non-histaminergic itching when injected into human skin binds to MrgX1, whereas β-alanine is a selective agonist of MrgD. Thus, members of the Mrg family serve as pruriceptors detecting different pruritogens on primary sensory neurons.

Other receptors involved in itch signalling have been protease activated receptors 2 and 4 (PAR2 and 4) which have been shown to be activated by cathepsin S or mucunain, the active ingredient of cowhage. Intradermal injection of SLIGRL-NH2 the unmasked N-terminus (tethered ligand) of PAR2, induced robust scratching behaviour in mice and itch sensation in humans. It was believed that this peptide is hydrolyzed by proteases and induces itching upon activating protease-activated receptor 2 (PAR2). However, intradermal injections of SLIGRL-NH2 exhibited comparable scratching behaviour in wild-type mice and PAR2 mutant mice, suggesting that PAR2 is not required for itch sensations mediated by SLIGRL-NH2. In contrast, Liu and colleagues could prove that SLIGRL-NH2 mediates itching by direct activation of MrgC11. This indicates that the liberated N-terminus of PAR2, after cleavage by a protease, is capable of activation MrgC11 if present in the same neuron or a neighbouring cell.

The Toll-like receptor (TLR) 3 has been implicated to play a role in pruritus. The TLR3 agonist polyinosinic-polycytidylic acid directly activated primary sensory neurons and evoked a scratching behaviour in a TLR3-dependent manner. Interestingly, TLR3--/ mice exhibited a reduced scratching behaviour not only for a specific TLR3 agonist but also for many other pruritogens, indicating that TLR3 may also be involved in mediating itch sensations in the central nervous system. Another toll like receptor, TLR7, has been shown to be expressed in sensory neurons. Two independent groups reported that imiquimod, a TLR7 agonist, induced scratching behaviour in mice. However, these groups presented conflicting data about whether the action of imiquimod is directly or indirectly mediated by TLR7.
Other important receptors in itch signalling represent the splicing variant of the \( \mu \)-opioid receptor (MOR), MOR1D, for morphine-induced pruritus,\(^\text{101}\) endothelin-A-receptor for endothelin-1,\(^\text{102}\) the interleukin-13 receptor for IL-13,\(^\text{103}\) and the heterodimeric receptor consisting of the IL-31 receptor \( \alpha \) (IL-31RA) and the oncostatin M receptor (OSMR) for IL-31.\(^\text{104}\) Activation of these receptors results in opening of transient receptor potential (TRP) receptors such as the vanilloid 1 receptor (TRPV1) or ankyrin 1 channel (TRPA1) on sensory neurons.\(^\text{79,105}\) Primary sensory neurons signal to the dorsal horn of the spinal cord where secondary neurons are activated by release of glutamate and neuropeptide natriuretic polypeptide b (Nppb).\(^\text{106}\) Secondary neurons express natriuretic peptide receptor A (NprA, the receptor for Nppb) and were suggested to release gastrin releasing peptide (GRP) which activates the GRP receptor of a third neuron in the spinal cord (see Figure 5, chapter 2).\(^\text{77,106,107}\) Ablation of either the NprA- or GRP-receptor expressing neurons by intrathecal application of a toxin bound to the respective signalling molecule largely abolished scratching behaviour after intradermal application of various pruritogens.\(^\text{77,106}\) Noteworthy, pain responses were unaltered after ablation of these neurons, indicating that a selective itch pathway exists on spinal cord level.\(^\text{77,106}\)

Together, these studies have revealed several receptors and signaling molecules as being involved mainly in acute forms of pruritus in mice and men. One major question which remains is the mechanism resulting in chronification of pruritus observed in many human disorders associated with pruritus. A recent mouse study shed light on this unresolved issue. Zhao and colleagues showed that constitutive activation of the serine/threonine kinase BRAF in neurons expressing the sodium channel Na\(_{\text{V}}\)1.8 was related to chronification of scratching behaviour.\(^\text{108}\) BRAF activated MEK1/2 which caused continuous phosphorylation of ERK resulting in increased expression of genes involved in itch signalling such as GRP, H\(_1\)-receptor or Mrg receptor A3.\(^\text{108}\) Thus, the RAF/MEK/ERK signalling cascade may represent a pharmacological target to ameliorate chronic itch sensation. Still, information on the role of specific pruritogens and mechanisms of chronification in human disorders remains very sparse.

It is common experience that intensity of pruritus may be temporarily affected by parenteral, oral or local application of a placebo. Hence, randomized, placebo-controlled
and double-blinded trials are needed to validate new antipruritic treatment strategies. Further unravelling of the pathogenesis of itch in cholestasis may help to develop novel more effective strategies among which possibly selective ATX inhibitors and LPA receptor antagonists. As ATX-LPA axis is involved in many physiological conditions it is likely that a general inhibition of this pathway by ATX inhibitors will be associated with many adverse effects. Once the LPA receptors involved in cholestatic pruritus are elucidated this problem may be overcome by administration of selective LPA receptor blockers.
REFERENCES


