SLC10A4 regulates IgE-mediated mast cell degranulation in vitro and mast cell-mediated reactions in vivo

Hanna Pettersson1,3, Behdad Zarnegar2, Annika Westin2, Viktor Persson2, Christiane Peuckert1, Jörgen Jonsson1, Jenny Hallgren2 & Klas Kullander1

Mast cells act as sensors in innate immunity and as effector cells in adaptive immune reactions. Here we demonstrate that SLC10A4, also referred to as the vesicular aminergic-associated transporter, VAAT, modifies mast cell degranulation. Strikingly, Slc10a4−/− bone marrow-derived mast cells (BMMCs) had a significant reduction in the release of granule-associated mediators in response to IgE/antigen-mediated activation, whereas the in vitro development of mast cells, the storage of the granule-associated enzyme mouse mast cell protease 6 (mMCP-6), and the release of prostaglandin D2 and IL-6 were normal. Slc10a4-deficient mice had a strongly reduced passive cutaneous anaphylaxis reaction and a less intense itching behaviour in response to the mast cell degranulator 48/80. Live imaging of the IgE/antigen-mediated activation showed decreased degranulation and that ATP was retained to a higher degree in mast cell granules lacking SLC10A4. Furthermore, ATP was reduced by two thirds in Slc10a4−/− BMMCs supernatants in response to IgE/antigen. We speculate that SLC10A4 affects the amount of granule-associated ATP upon IgE/antigen-induced mast cell activation, which affect the release of granule-associated mast cell mediators. In summary, SLC10A4 acts as a regulator of degranulation in vitro and of mast cell-related reactions in vivo.
Figure 1. SLC10A4 co-localises with mMCP-6 and lack of SLC10A4 does not interfere with the storage of mMCP-6 inside the mast cell granules. (A) Wild type and Slc10a4−/− BMMCs were immunostained with anti-SLC10A4 antibody (green), counterstained with DAPI (blue) and analysed by immunofluorescence microscopy. (B) Wild type BMMCs were stained with anti-SLC10A4 (green) and anti-mMCP-6 (red) antibodies and counterstained with DAPI to visualize granular staining. (B,C) The majority of the mast cell granules demonstrated co-localization (yellow) of mMCP-6 and SLC10A4 as indicated in the Venn diagram (analysis of...
112 wild type BMMCs. (D) Representative pictures of wild type (WT) and Slc10a4−/− BMMCs immunostained for mMCP-6 (red) and counterstained with DAPI (blue). As a negative control, BMMCs were stained with the secondary anti-goat antibody alone (control). (E) Quantitative western blot analysis of mMCP-6 expression in BMMCs derived from WT and Slc10a4−/− mice. The quantification analyses were performed by western blot on cell lysate and mMCP-6 expression levels were normalised to the β-actin amount in the same sample. The data shown are presented as mean ± SEM from six samples obtained from three independent western blots. No significant difference (p = 0.18) was found using a standard unpaired Student’s t-test. (F) WT and Slc10a4−/− bone marrow cells were cultured with SCF and IL-3 to obtain BMMCs. Samples from the indicated days after the start of the cultures in vitro (days in vitro; DIV) were cytopspun, stained with May–Grünwald/Giemsa and evaluated as positive or negative for cells with metachromatic staining. These data represent pooled data from two mice per genotype where the percentage of cells with metachromatic staining were calculated from the individual cell cultures based on three individual cytopspins per data point. Scale bars, 10 μm.

5-HT-receptors⁶,⁹. These mediators play essential roles in the inflammatory responses and as neurotransmitters in the nervous system.

The Slc10a4 gene encodes SLC10A4 also referred to as the vesicular aminergic-associated transporter, VAAT, which has primarily been associated to functionality of the aminergic systems.⁰ SLC10A4 was originally identified as an expressed sequence tag and was designated, based on sequence similarity, as a novel member of the solute carrier family 10 (SLC10) family.¹¹ The protein was cloned and characterized by Splinter and co-workers in 2006.¹² SLC10 is known as the sodium bile acid co-transporter family, since its seminal members, SLC10A1 (NTCP) and SLC10A2 (ASBT), are the bile acid transporters of the liver and the gut, respectively.¹³–¹⁵ In spite of these efforts, the substrate(s) of SLC10A4 still essentially remains unknown.¹⁶–¹⁸. Two studies have established that SLC10A4 is co-expressed with the carriers of acetylcholine (VACHT) and monoamines (VMAT2) on synaptic vesicles, both in the central and peripheral nervous systems.¹⁹–²⁰ This suggested the presence of SLC10A4 in other monoamine-containing secretory granules, which was supported by the identification of the SLC10A4 protein in rat peritoneal mast cells.²¹ While a role for SLC10A4 in the dopaminergic and cholinergic systems has been established, its role in mast cells has so far been unknown. In this study, we show that the SLC10A4 protein impacts the degranulation process of mast cells in vitro and regulates mast cell-mediated responses in vivo.

Results

We first set out to determine whether SLC10A4 was expressed by mouse mast cells. Bone marrow cells from Slc10a4-deficient and wild type mice were cultured in the presence of IL-3 and stem cell factor (SCF) to obtain c-kit+ FcεRI+ mast cells. Immunohistochemistry analyses demonstrated that SLC10A4 was expressed in BMMCs from wild type mice whereas the Slc10a4−/− BMMCs lacked staining of SLC10A4 protein (Fig. 1A). SLC10A4-specific immunoreactivity was previously demonstrated in secretory granules of rat peritoneal mast cells using immunohistochemistry and electron microscopy and found to be partly co-localized with VMAT2.²² In western blot experiments, SLC10A4 protein was enriched together with the synaptic vesicle protein synaptophysin throughout the purification steps of a rat brain vesicle preparation. Moreover, analysis of purified synaptic vesicles enriched from wildtype and knockout mice, confirmed expression of SLC10A4 localized to synaptic vesicles and absence of SLC10A4 in Slc10a4−/− mice.²³

SLC10A4 co-localises with mMCP-6 and the lack of SLC10A4 does not interfere with the storage of mMCP-6 in the mast cell granules. To further investigate the localisation of SLC10A4 protein in mast cells, wild type BMMCs were co-stained with antibodies toward SLC10A4 and the mast cell specific and granule localized protease, mMCP-6, and analysed by immunofluorescence microscopy. SLC10A4 immunopositive signals were found to overlap with mMCP-6 staining signals, suggesting an association of SLC10A4 with mouse mast cell granules (Fig. 1B). Quantification based on fluorescence signals showed that 88% of the fluorescence from SLC10A4 positively-stained granules overlapped with fluorescence from mMCP-6 positively stained granules. Conversely, 71% of the fluorescence from mMCP-6 positive granules overlapped with SLC10A4 positively-stained granules (Fig. 1C). Moreover, immune staining of mMCP-6 in wild type (age- and sex-matched littermate controls) and Slc10a4−/− mast cells suggested that the storage of mMCP-6 protein in the granules was intact in mast cells lacking SLC10A4 (Fig. 1D). Western blot analysis of mMCP-6 protein levels confirmed this observation (Fig. 1E).

To examine whether the lack of SLC10A4 was interfering with normal in vitro development of mast cells in the bone marrow cultures, wild type and Slc10a4−/− bone marrow cells were cultured in parallel and the maturation of mast cells was monitored. Cell samples taken from the cultures in regular intervals during the differentiation were stained with May–Grünwald/Giemsa and evaluated for their content of metachromatic mast cell granules. Overall, there were no differences in the staining properties or maturation into mast cells between wild type and Slc10a4−/− BMMCs (Fig. 1F). In addition, the BMMC cultures were routinely analysed by flow cytometry for c-kit and FcεRI expression before further experimentation. In these analyses, there were no differences in the proportion of c-kit+ FcεRI+ mast cells after three-four weeks of culture (Supplementary Fig. S1). Furthermore, a similar percentage of c-kit+ FcεRI+ peritoneal mast cells was observed in wild type and Slc10a4−/− mice using flow cytometry (Supplementary Fig. S2). In summary, SLC10A4 was found localized to the mast cell granules and lack of SLC10A4 did not interfere with the normal maturation process in vitro or the storage of mMCP-6 in the mast cell granules.
SLC10A4 is required for optimal IgE-mediated mast cell degranulation. We next tested whether SLC10A4 is involved in IgE/antigen-mediated mast cell degranulation. BMMCs were sensitized with IgE anti-TNP and treated with either OVA-TNP (IgE/ag), Ca²⁺-ionophore (A23187) or vehicle alone (Veh.). The data were obtained from three individual BMMC cultures from two mice per group, each treatment was performed in triplicates and analysed in quadruplicates. The data shown are from one representative experiment out of three. Asterisks mark significant differences (ns, non-significant, **P < 0.01, ***P < 0.001), Student’s t-test.
approximately 65% release of β-hexosaminidase whereas such IgE/antigen-mediated release of β-hexosaminidase from the Slc10a4−/− BMMCs was significantly reduced (Fig. 2A). Moreover, the histamine levels and tryptase activity were significantly reduced in the supernatants of Slc10a4−/− BMMCs after IgE/antigen stimulation compared with the wild type control BMMCs (Fig. 2B,C). Nevertheless, Ca2+-ionophore treatment, which causes unregulated release of virtually all granules, resulted in similar release of these granule-associated mediators in wild type and Slc10a4−/− BMMCs (Fig. 2B,C). We next set out to determine whether the SLC10A4-mediated reduction in mediators released after IgE/antigen stimulation was specific for granule-associated mediators or had a global effect on mediator release, and therefore quantified IL-6 and prostaglandin D2 levels in the supernatants after IgE/antigen-mediated activation. Both IL-6 and prostaglandin D2 were present at similar levels in the supernatants from wild type and Slc10a4−/− BMMCs after IgE/antigen-mediated activation (Fig. 2D,E). Together, these data suggest that SLC10A4 is involved in mast cell degranulation but not in the release of cytokines and lipid mediators after IgE/antigen-mediated activation.
Mast cells lacking SLC10A4 release less ATP in response to IgE-mediated degranulation. ATP is a granular component that may promote vesicular filling of monoamines, possibly by counterbalancing these positively charged molecules by the negatively charged ATP. To study whether the kinetic of the IgE/antigen-mediated degranulation process was modified in mast cells lacking SLC10A4, we next performed live cell imaging of cellular ATP. Briefly, BMMCs were stained with quinacrine, an acridine derivative known to bind ATP, before they were subjected to IgE/antigen-mediated activation. It has been demonstrated that the release of secretory granule content is associated with a decrease in quinacrine fluorescence in several different cell types. The quinacrine staining, which appeared granular in the raw images, was processed by the Imaris software from point type signals to spheres in order to calculate the volume. The fluorescent signals resulted in imaged and quantifiable volumes of the mast cells, which presumably reflects intragranular ATP levels.

Sensitized wild type and Slc10a4−/− BMMCs determined by the cellular fluorescent signals show similar ATP levels before activation (Supplementary Fig. S3). The fluorescent signals from wild type BMMCs were significantly reduced during the first 10 minutes after IgE/antigen-mediated mast cell activation as a result of a significant decrease in the calculated volume of the wild type mast cells after degranulation (Fig. 3A,B). The fluorescent signal of the wild type BMMCs remained at a similar level from 10 to 75 minutes post-activation, suggesting that the wild type mast cells were fully degranulated already after 10 minutes post-activation. In contrast, the Slc10a4−/− BMMCs had smaller calculated volume than wild type BMMCs immediately after IgE/antigen stimulation (t ∼0.5 min) (Fig. 3A,B and Table 1). Further, the cellular volume of Slc10a4−/− BMMCs remained at the approximately same level from 0.5 min until 65 min post-activation when there was a significant decrease in fluorescent signal compared to 0.5 min (Fig. 3A,B and Table 1). These results suggested that Slc10a4−/− mast cells, after a quick partial degranulation, degranulated very slowly. Furthermore, between 10–75 minutes post-activation, the cellular volume of wild type BMMCs remained smaller than the Slc10a4−/− BMMCs (Table 1), suggesting that mast cells lacking SLC10A4 never reached the same level of degranulation as their wild type counter parts.

We further quantified ATP in the supernatants that were analysed for mast cell mediators (depicted in Fig. 2) by a Luciferin-Luciferase bioluminescence assay one hour after IgE/antigen-mediated activation. Around three times more ATP was detected in the wild type BMMC supernatants compared to the supernatants from Slc10a4−/− BMMCs (Fig. 3C). This indicates that release of ATP upon IgE/antigen-mediated degranulation was strongly reduced in Slc10a4−/− mast cells. In addition, there was also less ATP detected in the supernatants from the Slc10a4−/− BMMCs after stimulation with a Ca2⁺-ionophore (Fig. 3C), suggesting that SLCT10A4-deficient mast cells stored less granular ATP. These data suggest that loss of SLCT10A4 led to a decreased ATP availability in mast cell granules, which resulted in a decreased release of granular ATP upon IgE/antigen-mediated mast cell activation.

Mice lacking SLC10A4 demonstrate reduced passive cutaneous anaphylaxis reactions. To determine if the decreased release of granule-associated mediators in mast cells lacking SLC10A4 would also have an effect on mast cell-related reactions in vivo, we turned to the IgE-mediated passive cutaneous anaphylaxis (PCA) model. This model evaluates mast cell-mediated allergic reactions, and more specifically, the immediate dermal response to mast cell activation that leads to release of mediators with increased permeability of vessels within the skin as a result. The response is visualized and quantified by Evans blue, which binds to and is extravasated with plasma albumin. To readily visualize the blue PCA reaction in less pigmented skin, Slc10a4−/− mice were backcrossed into the BALB/c background. Wild type control littermates and Slc10a4−/− mice were sensitized with anti-OVA IgE in the left ear and PBS in the right ear. Subsequent intravenous injection of OVA/Evans Blue in wild type controls resulted in a blue colour in the ears injected with anti-OVA IgE, but not in the PBS-injected ears (Fig. 4A,B). In contrast, Slc10a4−/− mice showed significantly attenuated blue colour extravasation in the anti-OVA IgE-injected ears after OVA/Evans Blue injection when compared to wild type controls (Fig. 4A,B). These data suggest that loss of SLC10A4 affected the release of histamine from mast cells in vivo.

Mice lacking SLC10A4 have a decreased scratching frequency after 48/80 injection. Within the epidermis, free nerve endings of non-myelinated C-type nerves mediate itch. A sub-population of these non-myelinated C nerve fibers respond specifically to histamine, predominantly through histamine H1 receptors.
but also through H4 receptors. Since mast cells reside in close proximity to blood vessels and nerves in the skin and store preformed histamine within their granules, mast cells are considered to play a central role in histamine-mediated itch reactions. We sought to determine whether SLC10A4 might play a role in mast cell-mediated reactions independent of the IgE/antigen activation pathway in vivo. For this purpose, wild type and Slc10a4−/− mice were given the mast cell degranulation compound 48/80 intradermally in the neck, as well as histamine or vehicle (saline) as positive and negative controls, respectively. Compound 48/80 induces mast cell degranulation through interaction with the G-protein coupled receptor Mrgprb2. All 48/80- or histamine-injected mice responded with increased scratching behaviour whereas vehicle injections did not induce an increased scratching behaviour, regardless of genotype (Fig. 4E). However, the pattern of scratching frequency and duration was different between the wild type and Slc10a4−/− mice upon 48/80 injections. While Slc10a4−/− mice in average had longer accumulated scratching time compared to wild type mice (Fig. 4C,D), Slc10a4−/− mice displayed significantly fewer scratch events than wild type controls (Fig. 4E). In contrast, histamine injections produced similar scratching responses in wild type and Slc10a4−/− mice, i.e. they displayed similar average scratching times (results not shown) and number of scratch events (Fig. 4E). Altogether, these data implicate SLC10A4 in 48/80-mediated scratch reactions.

**Discussion**

Cross-linking of IgE bound to the FcεRI by antigen causes strong mast cell activation, which results in the degranulation of mast cells and in the generation of lipid mediators and cytokines. Here, SLC10A4 in BMMCs was co-localized with a granule-associated protease and the loss of SLC10A4 in these cells led to a reduced release of granule-associated mediators after IgE/antigen-mediated activation. This implicates a role of SLC10A4 in the mast cell granules, which has a functional consequence during IgE/antigen-mediated degranulation.

The intracellular cascade of events that takes place in mast cells after IgE/antigen-mediated activation of FcεRI results in activation of src family kinases Lyn and Fyn and in the phosphorylation of downstream kinases, which phosphorylates immunoreceptor tyrosine-based motifs (ITAMs) on the β- and γ-chains of FcεRI. These events trigger calcium channels to open in the plasma membrane causing a receptor-operated calcium entry (ROCE).
that eventually lead to the activation of the IP$_3$ receptor on the endoplasmatic reticulum (ER), which stimulates Ca$^{2+}$ release from ER. This induces a conformational change in STIM1$^{33}$, which then translocates to specific junctions between ER and the plasma membrane and interacts with Orai1 and other calcium channel proteins to form Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channels$^{34,35}$. Calcium enters from the extracellular environment through such channels$^{36}$ and the increases in intracellular Ca$^{2+}$ regulate the release of lipid mediators in mast cells$^{38}$. SLC10A4-lacking mast cells were able to release normal levels of prostaglandin D2 in response to IgE/antigen-mediated activation, suggesting that calcium channel formation was normal. Furthermore, the normal IL-6 release after IgE/antigen-mediated cross-linking also suggests that the signalling pathways that regulates cytokine production and release were also intact in SLC10A4-deficient mast cells.

The increase in intracellular Ca$^{2+}$ levels can be accomplished externally by the addition of Ca$^{2+}$-ionophores like A23187, which was used as a positive degranulation control in this study. A23187 facilitates the increase in intracellular Ca$^{2+}$ levels by a non-receptor mediated mechanism through redistribution of intracellular Ca$^{2+}$ pools into the cytosol$^{37,38}$, which trigger degranulation through activation of protein kinase C (PKC)$^{39,40}$. Slc10a4$^{-/-}$ BMMCs showed a reduced release in granule mediators in response to IgE/antigen-mediated activation of FcεRI, whereas the release of granule mediators in response to Ca$^{2+}$-ionophore was essentially normal. This suggests that receptor-mediated signalling events were required to detect the influence of SLC10A4 on granule mediator release. Likely, the FcεRI-mediated mast cell activation cascade that ends with granule fusion is highly regulated, which is in contrast to the general activation by Ca$^{2+}$-ionophores such as A23187. Exactly how the early signalling events leading to Ca$^{2+}$ influx and the late signalling events monitoring exocytosis of granule components are coupled is unknown$^7$. The degranulation process, including exocytosis of granule-associated mediators, is regulated by SNAREs and is ATP-dependent$^{41,42}$. SNAREs, vesicle-associated membrane proteins involved in the docking and/or fusion of vesicles with the plasma membrane, have a similar function in the release of neurotransmitters from nerve cells. Because SLC10A4 is located in the mast cell granules and the storage of granule-associated mediators seemed intact in Slc10a4$^{-/-}$ BMMCs, it is possible that SLC10A4 influences the release of granule mediators by controlling the late events of mast cell degranulation. However, granule mediator release was intact after treatment with the Ca$^{2+}$-ionophore in the Slc10a4$^{-/-}$ BMMCs, thus, SLC10A4's function should then be controlled by signalling events triggered by receptor (FcεRI) activation. This warrants further studies of how the late signalling events differ between degranulation caused by activation via FcεRI and Ca$^{2+}$-ionophore A12387 in mast cells.

Mast cells contain one of the largest depts of histamine found in mammalian cells$^{43}$. Dopamine, like histamine, belongs to the monoamine neurotransmitter family and have similar requirements for their storage and uptake$^{44}$. Interestingly, studies of SLC10A4 in the nervous system have demonstrated that SLC10A4 can regulate the release and reuptake of dopamine, with behavioural deficits resulting from impaired dopamine homeostasis$^{45}$. Slc10a4$^{-/-}$ mice display increased sensitivity to amphetamine, and have lower brain levels of dopamine. The proposed mechanism behind the deficiencies in the central nervous system observed in Slc10a4$^{-/-}$ mice has been suggested to be the consequence of decreased vesicular monoamine re-uptake$^{46}$. The lower uptake may be caused by reduced presence of vesicular carrier ions in the synaptic terminals, such as ATP. Monoamines are positively charged at neutral pH. Therefore, to enter the more acidic vesicles, they need to overcome an electrical gradient in the form of protons$^{46}$. Similarly, the acidic environment in mast cell granules poses a challenge for granular uptake of positively charged histamine via VMAT2. In mast cell granules, highly negatively charged heparin and chondroitin sulphate E proteoglycans serve to counterbalance positive charge for protein and lipid mediators$^{47}$. These proteoglycans are also responsible for the staining of metachromatic mast cell granules, which occurs upon incubation with basic histological stains such as May–Grünwald/Giemsa. In our study, the in vitro differentiation of mast cells judged by the proportion of metachromatic granules was intact in the SLC10A4-deficient mast cells (Fig. 2E). This suggests that the storage of proteases and histamine, which depend on these negatively charged proteoglycans$^{48,49,50}$, were intact. In addition, western blot and confocal microscopy demonstrated a similar content of the granule localized protease mMCP6 in SLC10A4 deficient mast cells and their controls. However, for monoaminergic nerve cells, it has been proposed that ATP may act as a counter ion to alleviate an electrical gradient from the positively charged dopamine$^{45}$. Although the proteoglycans likely play a role in counteracting positive charges in mast cells, it remains possible that ATP could participate in this process. Interestingly, the levels of ATP in the supernatant after IgE/antigen-mediated degranulation of SLC10A4 lacking mast cells was only one third of the levels detected in the supernatant from wild type mast cells. Live cell imaging of IgE/antigen-mediated degranulation process demonstrated that the fluorescent signals originating from ATP localised to the granules of Slc10a4$^{-/-}$ BMMCs were retained to a higher degree than in wild type BMMCs post-activation. These data suggest that ATP contributed to the activation of the IP$_3$ receptor on the endoplasmatic reticulum (ER), which stimulates an increase in intracellular Ca$^{2+}$ pools$^{51}$, which trigger degranulation through activation of protein kinase C (PKC)$^{52,53}$. Slc10a4$^{-/-}$ mice demonstrated that the fluorescent signals originating from ATP localised to the granules of Slc10a4$^{-/-}$ mast cells in mice36. SLC10A4-lacking mast cells were able to release normal levels of prostaglandin D2 in response to IgE/antigen-mediated activation, suggesting that calcium channel formation was normal. Furthermore, the normal IL-6 release after IgE/antigen-mediated cross-linking also suggests that the signalling pathways that regulates cytokine production and release were also intact in SLC10A4-deficient mast cells.

The reduction in the release of granule-associated mediators in vitro translated to an effect on mast cell-mediated reactions in vivo. The PCA reaction in Slc10a4$^{-/-}$ mice was considerably reduced, suggesting that the release of histamine from mast cells lacking SLC10A4 was impaired in vivo. Further, itching, classically triggered by injection of compound 48/80 that leads to release of histamine from mast cells, was affected. Injection of compound 48/80 caused significantly less scratching events in the Slc10a4$^{-/-}$ mice than in the wild type mice. Control and SLC10A4-deficient mice responded similarly to an injection of histamine demonstrating a normal reaction to histamine in the skin of Slc10a4$^{-/-}$ mice. Even though the number of scratch events was fewer in the Slc10a4$^{-/-}$ mice, the time spent on scratching during each of the events (bout time) was longer than those of the controls. Possibly, the histamine-mediated scratching events were occurring so rapidly that a new scratch-event tended to occur before the first one naturally ended, resulting in shorter and faster scratching-behaviour in wild type mice. Alternatively, the injection of 48/80 also stimulates release of another SLC10A4-regulated mediator, which has a blocking effect on the scratch reaction mediated by the nerves. This would also lead to a prolonged
scratching behaviour in mice lacking SLC10A4. Altogether, the results from the PCA and itching experiments demonstrate that SLC10A4 regulates mast cell-related reactions in vivo.

In conclusion, our study for the first time identifies SLC10A4 as a regulator of mast cell degranulation. Our data imply that SLC10A4 affects the amount of granule-associated ATP upon mast cell activation with consequences for granule fusion with the plasma membrane, and therefore affects the efficiency of the release of granule-associated mast cell mediators. Thus, SLC10A4 is a potential novel drug target for mast cell-related diseases such as allergies.

Methods
Mice. SLC10A4 (Slc10a4−/−) deficient mice were generated in 129/SvEvBrd mice by Texas A&M Institute. These Slc10a4−/− mice were backcrossed to a C57BL/6 background for three generations and used in all experiments as sex-matched littersmates, except for the passive cutaneous anaphylaxis experiments. For these experiments, the third generation of Slc10a4−/− mice on C57BL/6 background were backcrossed to a BALB/c strain originally obtained from Bommice (Ry, Denmark) for nine generations before use. The mice used for experiments were bred in-house and all the experiments were performed following the rules and regulations of the Swedish Board of Agriculture and the National Veterinary Institute. All experiments were approved by the Stockholm Norra animal experiments ethics committee, Stockholm animal experiments ethics committee, Uppsala animal experiments ethics committee.

Culture of bone marrow-derived mast cells. The mice were euthanized by isoflurane overdose or neck dislocation. Bone marrow was harvested from femur and tibia by flushing the bones with RPMI-1640 complete medium (RPMI 1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES, 50 µM 2-ME, 1 mM sodium pyruvate, and 10% heat-inactivated FCS (all from Sigma-Aldrich)). Harvested cells were spun at 417 × g for 5 min at room temperature and the pellet was resuspended in RPMI-1640 complete medium enriched with 50 ng/ml each of SCF (Peprotech Nordic, Stockholm, Sweden) and IL-3 from the X63 supernatant. The number of viable cells was determined by trypan blue exclusion on a hemocytometer, adjusted to 5 × 10^5 cells/ml, twice per week and cultured in humidified 37 °C incubator with 5% CO₂. The percentage of FcεRI⁺ c-Kit⁺ mast cells were determined by flow cytometry on a LSR II (BD Biosciences, eBioscience, Hatfield, UK) using PE-Cy7 anti-c-Kit (2B8) and PE anti-FcεRI (MAR-1) antibodies (BD Biosciences, eBioscience, Hatfield, UK) after at least three weeks of culturing. The data was analysed using FlowJo (Tree Star Inc., Ashland, OR, USA). BMMC cultures containing 92–97% c-Kit⁺ FcεRI⁺ cells were used. In some experiments, the mast cell maturation was followed during in vitro culture. Samples from these cultures were taken in triplicates twice a week. The cells were cytospun onto glass slides (Shandon Cytopsin 2) and were allowed to dry overnight before staining by May-Grünwald/Giemsa (Sigma-Aldrich) using a standard protocol. The cells were imaged using a Nikon Eclipse Ni_U microscope, 400x magnifications. The software NSI-Elements BR 64-bit was used for capturing and editing, with automatic exposure time and medium contrast. All samples were scored blindly for presence or absence of fully matured granules within the cells during the developing period from the start of the culture to day 32 in vitro. A digital grid was applied to every picture and nine-squares/slide was analysed (at least 30 cells per slide were evaluated). The mean percentage of fully matured BMMCs was calculated from a total score per replicate/sample.

Confocal Microscopy. Approximately 5 × 10⁴ BMMCs of each genotype were cytospun (Shandon Cytopsin 2) at 500 rpm for six min to attach the cells onto glass slides. The cells were fixed with 4% paraformaldehyde for 10 min, followed by repeated washing with PBS (3 × 10 min). After blocking and permeabilization with 5% donkey serum and 0.1% Triton X-100 in PBS overnight at 4 °C, rabbit anti-SLC10A4 1:1000 (HPA028835, Sigma-Aldrich) and rat anti-mMCP-6 1:1000 (MAB3736, R&D Systems) were added in blocking solution. After staining for 10–12 hours at 4 °C, the cells were washed repeatedly and the secondary antibodies anti-rabbit-Alexa Flour 488 and anti-rat-Alexa Flour 647 (Jackson ImmunoResearch Europe LTD, UK) and DAPI (for nuclear counterstaining) were added. After one hour incubation at room temperature, the cells were washed and mounted with Mowiol (Sigma-Aldrich). A Zeiss laser point scanning confocal microscope, LSM 510 Meta (Carl Zeiss, Jena, Germany) was used. Image processing was performed with the software LSM 5 (Zeiss, Germany). Control staining for background fluorescence was kept in blocking solution until secondary antibodies were added. Immunohistochemistry co-staining, for expression of SLC10A4 and mMCP-6 were analysed by z-stack imaging by IMARIS 7.4 (Bitplane AG, Zurich, Switzerland).

In vitro stimulation assay. Mast cells were seeded at 1 × 10⁶ cells/ml and sensitized over night with anti-TNP IgE (prepared in-house from IgELB4⁻/⁻) at a final concentration of 2 µg/ml. The next day, cells were washed twice with PBS for removal of excessive IgE antibody and the cell pellet was resuspended in supplemented culture media (RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, 0.1 mM nonessential amino acids, 10 mM HEPES, 50 µM 2-ME, 1 mM sodium pyruvate, 20 g/l bovine serum albumin (A9312 BSA, Sigma) and 50 ng/ml of each SCF and IL-3). The cell concentration was adjusted to 1 × 10^6 cells/ml before seeding to a 24-wells plate. For activation, the following reagents were added to obtain a final concentration of 100 ng/ml OVA-TNP, 2 µM Ca²⁺-ionophore (A23187) or vehicle (supplemented culture media). After one or 18–24 h activation (for IL-6 release), 600 µl cell suspension from each well were transferred to tubes and centrifuged for 500 × g for 5 min at 4 °C. The supernatant and cell fraction were analysed for presence of mediators either directly or after snap freezing and storage at −80 °C until analysis.

Quantifications of mast cell mediators. For quantification of β-hexosaminidase, mast cell supernatants were collected directly after in vitro stimulation and cell pellets were lysed. To assay for retained
granulated molecules, 500 μl 1.2% freshly prepared Triton-X100 was added and vortexed briefly. Sixty μl supernatant and cell lystate was added in triplicates to a 96-well ELISA plate and incubated for two hours at 37 °C with 60 μl of 4-nitrophenyl-N-acetyl-β-D-glucosaminide (N9376, Sigma) [3 mg/ml in 80 mM citric acid buffer]. The reaction was quenched by adding 120 μl of L-lysine [0.2 M] and the reaction analysed by absorbance measurement at 405 nM (Versamax microplate reader, Molecular Devices). Supernatants from activated mast cells were also analysed by the following kits: Histamine (BA E-5800; Labor Diagnostika Nord GmbH, Germany), Prostaglandin D2 (MBS262231; MyBioSource, San Diego, USA), ATP ( Luciferin-Luciferase Bioluminescence assay (FL- AA, Sigma)) and IL-6 (CA92121, 88-7064-88; eBioscience Inc., San Diego, USA) according to the manufacturer guidelines. For tryptase activity measurements, 20 μl of fresh supernatant was diluted with 80 μl PBS and 20 μl of the substrate S-2288 (28083239, Chromogenix, NY) was added. Immediately thereafter, the absorbance was measured every 30 sec for 45 min at 405 nm and the Vmax expressed as mOD/min determined (Versamax microplate reader, Molecular Devices).

**Live cell imaging of mast cells upon IgE/antigen-induced mast cell activation.** For analysis of the time course of mast cell degranulation, approximately 5 × 10^4 cells were stained with 20 nM quinacrine (Sigma) for 10 min in humidified 37 °C incubator with 5% CO₂. After washing twice with 37 °C pre-warmed PBS, cells were resuspended in 150 μl of supplemented media and seeded to a 35 mm glass bottom microwell dish (P35GC-1.5-10-C, MatTek Corporation, MA, USA). BMMCs were either stimulated with 100 ng/ml of OVA-TNP to initiate degranulation or left untreated to evaluate impact of auto bleaching under the microscope. Degranulation was monitored by time-lapse on a confocal microscope (Zeiss LSM 510 Meta instrument; 63x magnification, pinhole 1.4 and 1.29 air unit). Z-stack images of 10 pictures/stack and 4 scans/slide were taken every 30 sec over a period of 75 min. Imaging analysis was done with IMARIS 7.4 (Bitplane AG, Zurich, Switzerland).

**Western blot.** Slc10a4⁻/⁻ and wild type BMPCs were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, all from Sigma) supplemented with complete protease inhibitor cocktail (Roche) and total protein concentrations determined. Cell lysates were stored at −20 °C until analysis. Twenty μg BMPC lystate was separated on 4–15% mini-PROTEAN® TGX™ precast gel (Bio-Rad Laboratories AB, Sweden) and transferred to a nitrocellulose membrane (Bio-Rad) in a semi-dry electrophoretic transfer cell (Bio-Rad). The membrane was blocked with 2% BSA in TBST (50 mM Tris pH 8, 150 mM NaCl, 0.05% Tween® 20) and incubated with primary antibody in the same blocking solution for 16 h at 4 °C, followed by four washes in TBST. The membrane was then incubated with secondary antibody conjugated to horse radish peroxidase for 1 h at room temperature followed by washing in TBST. The protein bands were detected with ECL-prime luminol reagent (Pierce™, ThermoFisher Scientific) in a gel documentation system (Chemi-Doc, BioRad). Antibody dilutions were: rabbit-anti-SLC10A4, (1:5000 from Novus Biologicals, Colorado, USA), mouse-anti rabbit-HRP 1:10,000 (Cat: 211-032-171, Jackson lab), goat-anti rabbit-anti-IgG 1:2000 (HPA028835, Sigma), rat-anti-mMCP-6 1:2000 (MAB3736, R&D Systems) mouse-Actin β1:5000 (Cat: 377331, Sigma). Antibody dilutions were: rabbit-anti-SLC10A4, 1:2000 (HPA028835, Sigma), rat-anti-mMCP-6 1:2000 (MAB3736, R&D Systems) mouse-Actin β1:5000 (Cat: 377331, Sigma). Western blotting analyses of the western blot results were obtained using ImageJ.

**Passive cutaneous anaphylaxis.** Mice were anesthetized and injected intradermally in the left ear with 10 μl [1 mg/ml] anti-OVA IgE prepared in-house from the To: hybridoma in PBS and 10 μl PBS in the right ear, as internal control. After 24 h, the sensitized mice were given 200 μl OVA containing 0.5% Evans Blue in PBS [0.25 μg/μl] intravenously in the tail and the animals were euthanized after 20 min and the ears removed for colorimetric analysis. The ears were weighed to calculate normalization, and the extravasation of Evans Blue was analyzed by addition of KOH [1 M] 37 °C on shake overnight and neutralized by adding 0.2 M H₃PO₄:acetone (5:13, v/v). The digested ear solution was spun twice at 16000 rcf for 10 min to remove cellular and tissue debris. Absorbance was measured in triplicate samples at 620 nm and normalized against tissue weight.

**Itch assay.** All behavioural tests were performed on adult (>7 weeks old) male Slc10a4⁻/⁻ mice and their wild type littermates in a controlled environment of 20–24 °C, 45–65% humidity and 12 hours day/night cycle. The observers were blind to the genotype. The mice were injected intradermally in the neck with 50 μl 48/80 or histamine [2 μg/μl] in saline (9% physiological NaCl) or as basal control animals were injected with 50 μl of saline as vehicle. One hour video recordings were used to quantify scratch behaviour, and were later scored blindly for analysis of individual behaviour patterns.

**Statistical analysis.** The software GraphPad 5 was used for statistical analysis. Significance definitions were set as follows, *P < 0.05, **P < 0.01 and ***P < 0.001 are represented by *, ** and *** Respectively.

**References.**

1. Galli, S. J. & Tsai, M. IgE and mast cells in allergic disease. *Nat Med* 18, 693–704, doi:10.1038/nm.2755 (2012).
2. Blank, U. et al. Vesicular trafficking and signaling for cytokine and chemokine secretion in mast cells. *Front Immunol* 5, 453, doi:10.3389/fimmu.2014.00453 (2014).
3. Wernersson, S. & Pejler, G. Mast cell secretory granules: armed for battle. *Nat Rev Immunol* 14, 478–494, doi:10.1038/nri3690 (2014).
4. Forsberg, E. et al. Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. *Nature* 400, 773–776, doi:10.1038/23488 (1999).
5. Humphries, D. E. et al. Heparin is essential for the storage of specific granule proteases in mast cells. *Nature* 400, 769–772, doi:10.1038/23481 (1999).
6. Hallgren, J., Backström, S., Estrada, S., Thuveson, M. & Pejler, G. Histidines are critical for heparin-dependent activation of mast cell tryptase. *J Immunol* 173, 1868, doi:10.4049/jimmunol.173.3.1868 (2004).
7. Merickel, A. & Edwards, R. H. Transport of histamine by vesicular monoamine transporter-2. *Neuropharmacology* 34, 1543–1547, doi:10.1016/0028-3908(95)00114-Y (1995).
8. Holst, S. C., Valomon, A. & Landolt, H. P. Sleep Pharmacogenetics: Personalized Sleep-Wake Therapy. *Annu Rev Pharmacol Toxicol* 56, 577–603, doi:10.1146/annurev-pharmtox-010715-103801 (2016).
14. Hagenbuch, B., Stieger, B., Foguet, M., Lubbert, H. & Meier, P. J. Functional expression cloning and characterization of the hepatic bile salt cotransporter Na+/bile acid cotransporter in Xenopus laevis oocytes. *J Biol Chem* 265, 5357–5360 (1990).

15. Wong, M. H., Oelkers, P., Craddock, A. L. & Dawson, P. A. Expression cloning and characterization of the hamster ileal sodium-bile acid cotransporter. *Proc Natl Acad Sci USA* 88, 10629–10633, doi:10.1073/pnas.88.23.10629 (1991).

19. Burger, S. et al. Co-expression studies of the hamster carrier protein Slc10a4 and the vesicular carriers VACHT and VMAT2 in the rat central and peripheral nervous system. *Neuroscience* 152, 990–1005, doi:10.1016/j.neuroscience.2008.01.049 (2008).

23. Breckenridge, L. J. & Almers, W. Final steps in exocytosis observed in a cell with giant secretory granules. *J Biol Chem* 269, 1340–1347 (1994).

26. Patra, K. A role for soluble carrier family member 10 in vesicular amine-associating transport, in structural re-modelling and transmitter release at the mouse neuromuscular junction. *Eur J Neurosci* 41, 316–327, doi:10.1111/j.1460-9568.2015.08562.x (2015).

29. Raap, U., Stander, S. & Metz, M. Pathophysiology of itch and new treatments. *Clin Immunol* 152, 89–96, doi:10.1016/j.clim.2013.09.015 (2013).

31. Inesi, G. & Tadini-Buoninsegni, F. Ca(2++)/H (+) exchange, luminal Ca(2+) release and Ca(2+)/ATP coupling ratios in the sarcoplasmic reticulum ATPas. *J Cell Commun Signal* 3, 101–112, doi:10.1007/s12078-012-0020-3 (2008).

33. Prakriya, M. The Ca(2+) fluxes through store-operated channels may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell Calcium* 33, 101–112, doi:10.1016/j.cacal.2007.03.007 (2008).

37. Abramov, A. Y. & Duchen, M. R. Actions of ionomycin, 4-BrA23187 and a novel electrogenic Ca(2+) release by mast cell granules. *Nat Immunol* 7, 1340–1347, doi:10.1038/ni817 (2006).

40. Kim, D. Y. et al. Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat Immunol* 9, 89–96, doi:10.1038/nature02800 (2008).

43. Bergendorff, A. Intracellular distribution of amines taken up by rat mast cells. *Acta Physiol Scand* 95, 133–141, doi:10.1111/j.1748-1716.1975.tb0035x (1975).

45. German, C. L., Balah, M. G., McFadden, I. M., Hanson, G. R. & Fleckenstein, A. E. Regulation of the Dopamine and Vesicular Monoamine Transporter: Pharmacological Targets and Implications for Disease. *Pharmacol Rev* 67, 1005–1024, doi:10.1124/pr.114.010397 (2015).

47. Rudolph, A. K., Burrows, P. D. & Wahl, M. R. Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Iga or Igb heavy chain haplotype. *Eur J Immunol* 21, 527–529, doi:10.1002/eji.18002101671 (1991).

51. Sawada, K. et al. The expression of murine cutaneous late phase reaction requires both IgE antibodies and CD4 T cells. *Clin Exp Allergy* 27, 225–231, doi:10.1111/cea.1997.27.issue-2 (1997).

57. Rudolph, A. K., Burrows, P. D. & Wahl, M. R. Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Iga or Igb heavy chain haplotype. *Eur J Immunol* 21, 527–529, doi:10.1002/eji.18002101671 (1991).

60. Kasuya, H. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using high-copy cDNA expression vectors. *Tanjakushitu Kakusan Koso* 33, 2527–2532 (1988).

63. Rudolph, A. K., Burrows, P. D. & Wahl, M. R. Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Iga or Igb heavy chain haplotype. *Eur J Immunol* 21, 527–529, doi:10.1002/eji.18002101671 (1991).
Acknowledgements
This work was supported by grants from the Swedish Research Council (J.H., K.K.) and Malin and Lennart Phillipson Foundation (J.H.), and the Hällsten foundation (K.K.). We thank Joakim Dahlin and Kalicharan Patra for technical input. Flow cytometric analyses were performed on equipments provided by the BioVis Facility at the Science for Life Laboratory, Uppsala, Sweden.

Author Contributions
H.P. and B.Z. grew and analysed the BMMC cultures; V.P. and H.P. performed the BMMC maturation experiments; H.P. performed the in vitro activation experiments; H.P. and C.P. performed the confocal microscopy; H.P., B.Z. and A.W. performed the PCA experiments; H.P. performed the in vivo scratch experiment; J.J. initiated the collaboration and had input on the project design; H.P., B.Z., J.H. and K.K. analysed the data; H.P., J.J., K.K. and J.H. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-01121-8

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

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017