Expression and role of serotonergic and nicotinic acetylcholine receptors in alveolar macrophages

Zbigniew Mikulski

INAUGURALDISSERTATION
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IV. Abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
5-HT	5-hydroxytryptamine, serotonin
ACh	acetylcholine
AM	alveolar macrophages
APC	allophycocyanin
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CarAT	carnitine acetyltransferase
ChAT	choline acetyltransferase
DAG	diacylglycerol
DRG	dorsal root ganglia
DTT	dithiothreitol
EDTA	ethylendinitrilo-N, N, N´, N´, -tetra-acetic acid disodium salt
EGTA	ethylene glycol-bis (2-amino-ethylether)-N,N,N',N'–tetra-acetic-acid
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
G-CFU	granulocyte colony forming unit
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GM-CFU	granulocyte/macrophage colony forming unit
GPCR	G-protein-coupled receptors
HPRT1	hypoxanthine guanine phosphoribosyl transferase 1
HSC	hematopoietic stem cell
IFN	interferon
IL	interleukin
IM	lung interstitial macrophages
$Ins(1,4,5)P_3$	inositol-1,4,5-trisphosphate
$Ins(1,4,5)P_3R$	inositol-1,4,5-trisphosphate receptor
ITAM	immunoreceptor tyrosine-based activation motif
LPS	lipopolisacharyde
mAb	monoclonal antibody

M-CFU	macrophage colony forming unit
M-CSF	macrophage colony stimulating factor
nAChR	nicotinic acetylcholine receptors
NCX	Na^+/Ca^{2+} exchanger
NF-κB	nuclear factor kappa B
PBS	Dublecco's phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PE	phycoerythrin
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PM	peritoneal macrophages
PMCA	plasma-membrane Ca^{2+}-ATPase
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
RYR	ryanodine receptor
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SERT	serotonin reuptake transporter
SOCE	store-operated calcium entry
SR	sarcoplasmic reticulum
TNF	tumor necrosis factor
TPH	tryptophan hydroxylase
VAChT	vesicular acetylcholine transporter
β-MG	beta microglobulin

1 Introduction

1.1 *Macrophages*

Macrophages are indispensible in the maintenance of tissue homeostasis, through the initiation of inflammatory responses to invading pathogens, clearance of senescent cells, organ modeling and wound healing [1]. Macrophages are present in large numbers lining lamina propia of a mucosa, pulmonary alveoli, juxtaglomerular and perivascular spaces, bony trabeculae, and renal tubules, where they show high a degree of heterogeneity which reflects the specialization of function adopted by these cells at different anatomical locations. Macrophage-like cells appear early during ontogenesis in the yolk sac [1]. Later in development, macrophages resembling those that are present in adults are generated in large numbers in the haematopoietic fetal liver, from where they colonize most organs [1] (Fig. 1.1). The current classification of phagocytic cells operates in a framework of the mononuclear phagocyte system, which includes macrophages and their monocyte precursors and lineage-committed bone-marrow precursors. Although monocytes can serve for the replenishment of tissue-resident macrophage populations, there is solid evidence for self-renewal of macrophages in many peripheral organs, e.g. in the skin [2] or lung [3, 4].

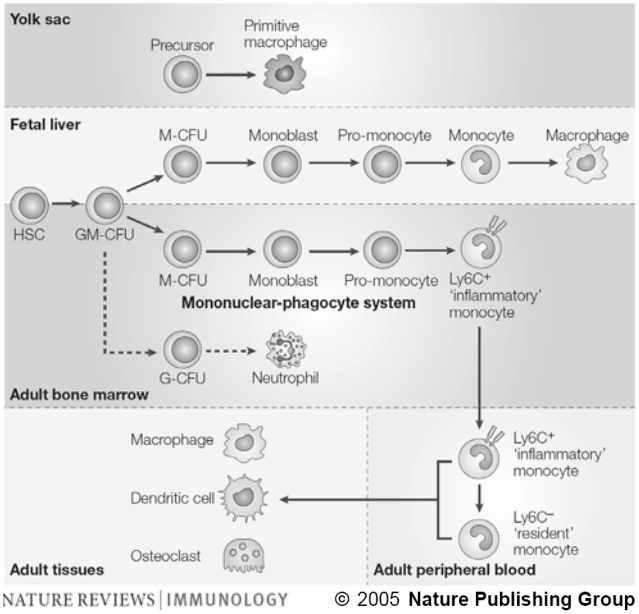

Fig. 1.1 Development of the mononuclear phagocyte system.

Adapted by permission from Macmillan Publishers Ltd: Nature Reviews: Immunology, Gordon S. and Taylor P.R. [1]. M-CSF = macrophage colony stimulating factor, HSC = hematopoietic stem cell, GM-CFU = granulocyte/macrophage colony forming unit, M-CFU = macrophage colony forming unit, G-CFU = granulocyte colony forming unit

1.2 *Origin of alveolar macrophages*

In the lung, macrophages are present in two distinct compartments: lung interstitium and alveolar region. It has been suggested that lung interstitial macrophages (IM) are intermediate between blood monocytes and alveolar macrophages (AM) [4]. Inflammatory monocytes, which carry CCR2 receptors and Ly6C molecules on their surface, are recruited and differentiate into macrophages at the site of inflammatory lesions. This was confirmed by experiments in which CCL2, the ligand of CCR2 receptors, was deposited into the lung [5]. CCL2 is the major chemokine that drives monocyte recruitment in bacterial [6] and viral [7] models of lung inflammation. The hypothesis of self-renewal of AM has been addressed in animal models and human subjects undergoing bone marrow transplantation. If lungs of irradiated mice, which subsequently received bone marrow transplantation, were protected from radiation, animals retained most of the recipient AM 1 year after the treatment [1]. However, after whole-body irradiation and transfer of GFP-labeled bone marrow, host AM were replaced with cells of donor origin, indicating that AM can be replenished from the bone marrow [1]. This was further refined in experiments in which the resident lung mononuclear phagocytes were depleted by diphtheria toxin. After bone marrow transplantation, phagocytic cells that recolonized the lung tissue were identified as blood derived monocytes, which then moved into the interstitium, acquired macrophage-like phenotype and finally reached alveoli [4]. Moreover, it has been shown by staining with antibodies detecting PCNA (proliferating cell nuclear antigen) that IM and AM proliferate follow depletion [4]. Studies on humans who have received allogeneic bone-marrow transplants support this notion and indicate that this replenishment occurs by recruitment of precursors, followed by proliferation of macrophages *in situ* [3, 8]. It is postulated that replenishment of AM in non-inflammatory conditions is dependent on monocytes which lack the expression of CCR2 and Ly6C molecules, but upregulate CX_3CR1 receptors [1].

1.3 *Function of alveolar macrophages*

Lung macrophages represent the first line of defence to inhaled environmental toxins and pathogens. In the stady state condition, the leukocyte population in the alveolar space is dominated by AM (90%), the reminder being mainly dendritic cells and T cells [9]. The major function of AM is phagocytosis and sequestration of antigen from the immune system to shield the alveolus from the development of specific immune response [9], thus ensuring proper gas exchange. High levels of expression of surface receptors, including pattern

recognition receptors such as scavenger receptors, mannose receptors, and the β-glucan specific receptor [10], allow AM to vigorously phagocytose material which was deposited into the lung. It is unclear if AM transport antigens to regional lymph nodes and have a significant role in antigen presentation. Previous data suggested that lung antigens are exclusively transported in a CCR7-dependent fashion by dendritic cells [11], but recent work by Kirby *et al.* showed that murine AM constitutively migrate from the lung to draining lymph nodes, and following exposure to *Streptococcus pneumoniae*, AM rapidly transport bacteria to this site [12]. The potential role of these migrating AM in antigen presentation remains elusive. Interestingly, AM actively suppress the induction of adaptive immunity in the lung. Studies with clodronate-filled liposomes which selectively deplete AM when given intratracheally, showed that treated rodents were susceptible to T cell-mediated inflammatory responses to otherwise harmless antigens [9]. This phenomenon was attributed to the enhanced antigen presentation by dendritic cells, indicating that AM might be inhibitory upon antigen presenting cells.

In the steady state condition, AM are maintained in a quiescent state and produce small amounts of pro-inflammatory cytokines. Equipped with surface receptors for numerous ligands, AM can respond to environmental factors, including cytokines, microorganisms and particulate material with the secretion of a large number of products [9]. Activation of AM results in a shift in their functional capacity to phagocytosis [13], microbial killing [14], and initiating immune response by secreting cytokines like TNFα and IL-1β which are essential for activation of the epithelium and downstream inflammatory responses [15]. Moreover, AM produce chemokines such as CCL2 and CCL5, which recruit activated monocytes and lymphocytes into sites of inflammation in the lungs [16].

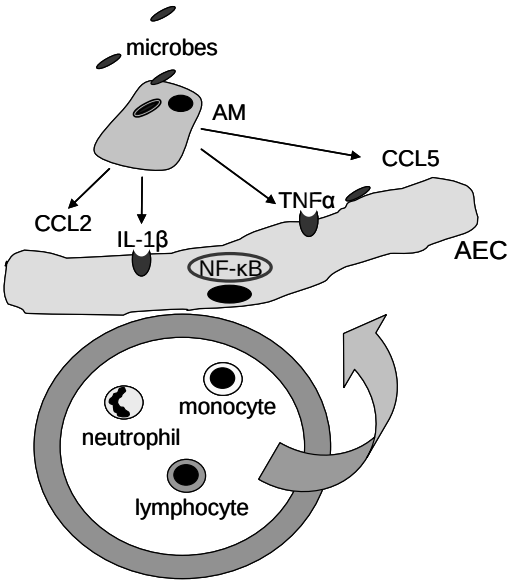

Fig. 1.2 Initiation of inflammatory response by alveolar macrophages.
Alveolar macrophages recognizing microbes activate epithelial cells by secreting TNFα and IL-1β. These diverse activation pathways converge on nuclear factor (NF-κB), which drives transcription of proinflammatory mediators, including neutrophil chemokines, colony-stimulating factors, and adhesion molecules. Additionally, AM produce chemokines (CCL2, CCL5) which recruit activated monocytes and lymphocytes into sites of inflammation in the lungs. AM = alveolar macrophages, AEC = alveolar epithelial cells, IL-1β = interleukin 1β TNFα = tumor necrosis factor α.

1.4 *Paracrine and autocrine signaling in macrophages*

As it has been indicated in the previous section, AM actively sense and signal to their environment. This communication is rarely achieved by direct cell contact; more often cells secrete and respond to soluble mediators, i.e. chemokines and cytokines. Signaling by extracellular, secreted molecules can be classified into three types — endocrine, paracrine, or autocrine, based on the distance between the source of signal and the receiver. Cells are equipped with vast array of receptors linked with intracellular signaling pathways that allow them to receive and process signals. Cell surface receptors on macrophages important for this signaling include ligand-gated ion channels and G-protein-coupled receptors (GPCR).

1.4.1 Ligand-gated ion channels

The nicotinic acetylcholine receptors (nAChR) are prototypic ligand-gated ion channels, which all share similar architecture and function [17]. Parts of the transmembrane domains of the receptor subunits create a hydrated pore that is also permeable to selected ions (Fig. 1.3). Since ion-channel receptors reside in a constant equilibrium between open and closed states, receptors contain components that are responsive to and regulated by the presence of external compounds such as activators (agonists), inhibitors (antagonists), or compounds that modify the efficacy of these agents [17]. Conformation of the receptor changes upon agonist binding from hydrophobic-based, channel closed configuration to a more open, hydrophilic channel that favors the ion passage [17]. Role and function of nAChR in immune cells will be discussed in further details in the section 1.6.

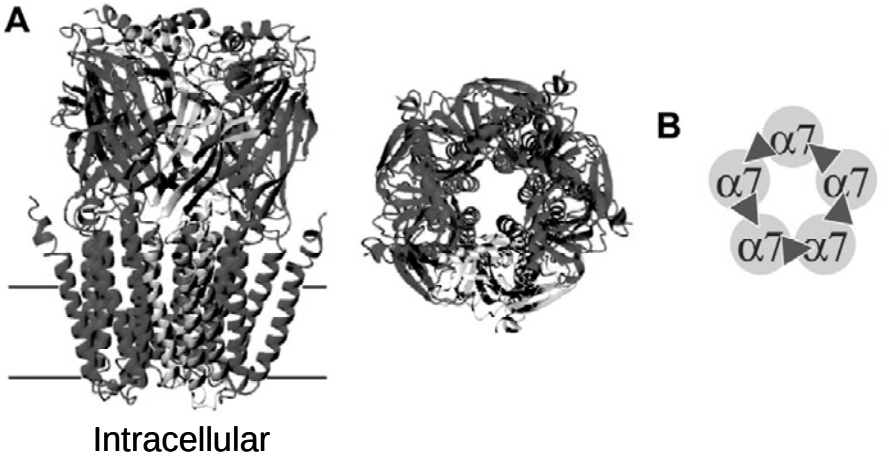

Fig. 1.3 Structure of the nAChR.

Adapted and modified from Cloëz-Tayarani I. and Changeux J.P. 2007 [18] (A) Three-dimensional, computerized model of the pentameric α7 nAChR (B) Schematic drawing illustrating positions of ligand binding pockets (triangles) at the interfaces between the helices.

1.4.2 G-protein-coupled receptors

GPCR, also known as seven-transmembrane domain receptors, constitute the largest known superfamily of cell-surface receptors with ~1000 distinct receptors encoded in the human genome [19]. Despite their diversity, all GPCR mediate their effects, at least in part, through coupling to heterotrimeric G proteins [19], comprising α, β, and γ subunits. The α subunit is responsible for GTP and GDP binding and for GTP hydrolysis, whereas the β and γ subunits

are associated in a tightly linked βγ complex [20]. Activation of a GPCR triggers conformational change that leads to G protein activation by exchange of bound GDP to GTP (Fig. 1.4). The activated heterotrimer dissociates into an α subunit and a βγ dimer, both of which have an independent capacity to regulate separate effectors (Tab. 1.1) [20]. Upon agonist activation of receptors, a rapid attenuation of receptor responsiveness occurs through feedback mechanisms, which involve phosphorylation of activated GPCR by GPCR kinases and/or second messenger-dependent kinases [19].

G-protein subunit	Function
$G\alpha_s$	↑Adenylyl cyclase, increase in cAMP levels
$G\alpha_i$	↓Adenylyl cyclase, decrease in cAMP levels
$G\alpha_q$, $G\alpha_{11}$	↑Phospholipase C, increase in Ins(1,4,5)P_3 and DAG levels
$G\alpha_{12}$, $G\alpha_{13}$	↑Rho-GEF, ↑Rho, induction of stress-fiber formation
Gβγ	↑Adenylyl cyclase, ↑Phospholipase C, increase in cAMP and Ins(1,4,5)P_3 levels

Tab. 1.1 Examples of heterotrimeric G-protein mediated effector functions.
Based on Pierce K.L, Premont R.T. and Lefkowitz R.J. [20]. ↑ denotes activation, ↓ denotes inhibition, cAMP = cyclic adenosine monophosphate, Ins(1,4,5)P_3 = inositol-1,4,5-trisphosphate, DAG = diacylglycerol, GEF = guanine nucleotide exchange factor

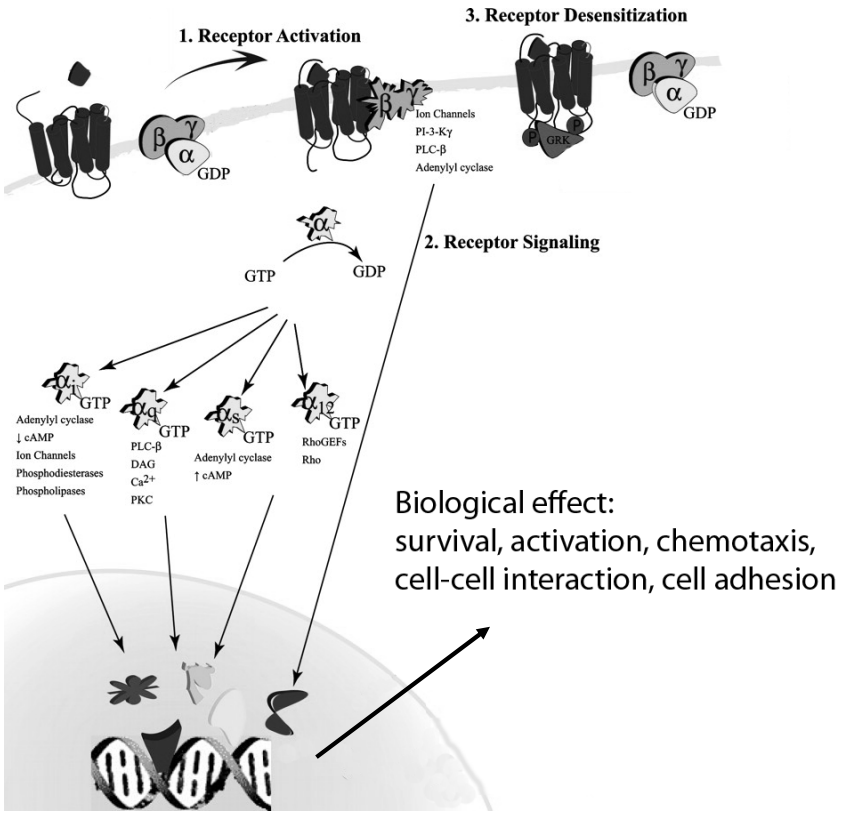

Fig. 1.4 GPCR signaling.

Adapted and modified from Lattin *et al.* [19]. Ligand binding induces conformational changes in the receptor, which binds G-proteins and promotes its activation. The GTP-bound α-subunit dissociates from the βγ-subunits; the free subunits regulate effector proteins which translate receptor stimulation to the biological effects. Signaling through GPCR is terminated by receptor desensitization and internalization.

1.5 *Role of Ca^{2+} in regulation of macrophage function*

1.5.1 Calcium homeostasis in immune cells

Ca^{2+} is a highly versatile intracellular signal that can regulate many different cellular functions over a wide dynamic range [21]. The level of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is determined by a balance between the 'on' reactions that introduce Ca^{2+} into the cytosol and the 'off ' reactions through which this signal is removed by the combined action of buffers, pumps and exchangers [21]. Duration, amplitude and spatio-temporal patterning of $[Ca^{2+}]_i$ signal control various cellular activities, from exocytosis, which starts within microseconds after Ca^{2+} rise, to gene transcription that requires several minutes to hours of prolonged $[Ca^{2+}]_i$ elevation [22] (Fig. 1.5).

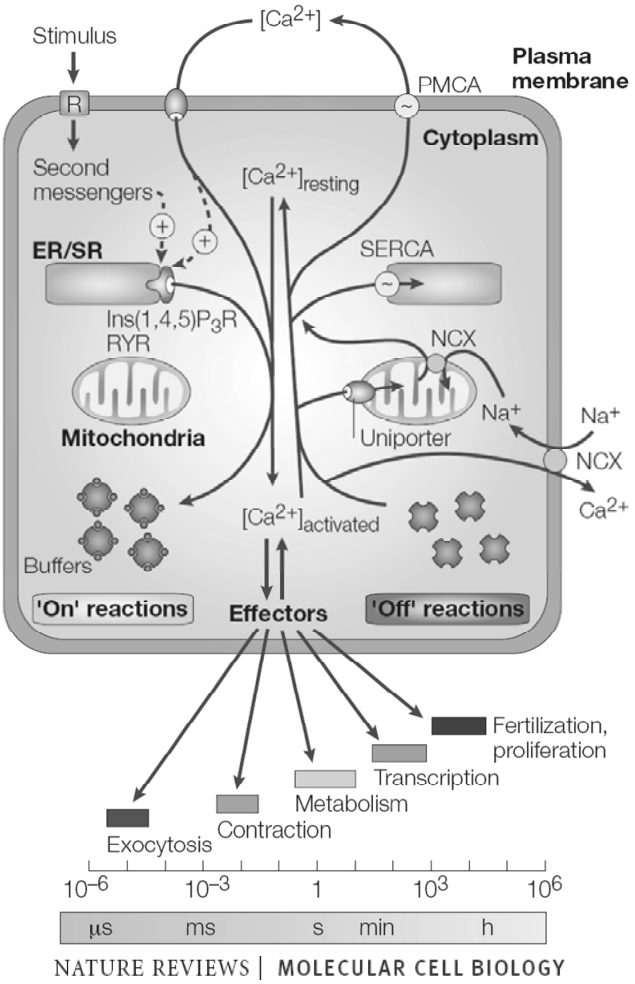

Fig. 1.5 Calcium-signaling dynamics and homeostasis.

Adapted by permission from Macmillan Publishers Ltd: Nature Reviews: Molecular Cell Biology, Berridge M.J., Bootman M.D and Roderick H.L [22]. Different stimuli induce entry of external Ca^{2+} and the formation of second messengers that liberate Ca^{2+} from intracellular Ca^{2+} stores. Most of this Ca^{2+} (red circles) is bound to buffers, whereas a small proportion binds to the effectors that activate a variety of cellular processes in spatio-temporal fashion. During the “off' reactions” Ca^{2+} is removed from cytosol by various exchangers and pumps. NCX = Na^{+}/Ca^{2+} exchanger, PMCA = plasma-membrane Ca^{2+}-ATPase, SERCA = sarco(endo)plasmic reticulum Ca^{2+}-ATPase, ER/SR = endoplasmic/sarcoplasmic reticulum, $[Ca^{2+}]$ = Ca^{2+} concentration, Ins(1,4,5)P_3R = inositol-1,4,5-trisphosphate receptor, RYR = ryanodine receptor.

Both external and internal sources of Ca^{2+} are utilized by immune cells to initiate their Ca^{2+} signals (Fig. 1.6). In macrophages, extracellular Ca^{2+} influx can be triggered by agonist binding to ligand-gated ion channels, e.g. purinergic P2X receptors [23]. However, the

predominant pathway of increase in $[Ca^{2+}]_i$ is thought to involve formation of second messengers and release of Ca^{2+} from the intracellular stores within the membrane systems of the endoplasmic reticulum (ER) [22, 24]. Various channels like the inositol-1,4,5-trisphosphate receptor and ryanodine receptor release Ca^{2+} from these internal stores [25, 26]. In immune cells, the seven transmembrane receptors which couple to G_q/G_{11}-proteins, and immunoreceptors containing immunoreceptor tyrosine-based activation motif (ITAM) are two important classes of proteins which activation leads to release of Ca^{2+} from intracellular stores (Fig. 1.6) [22].

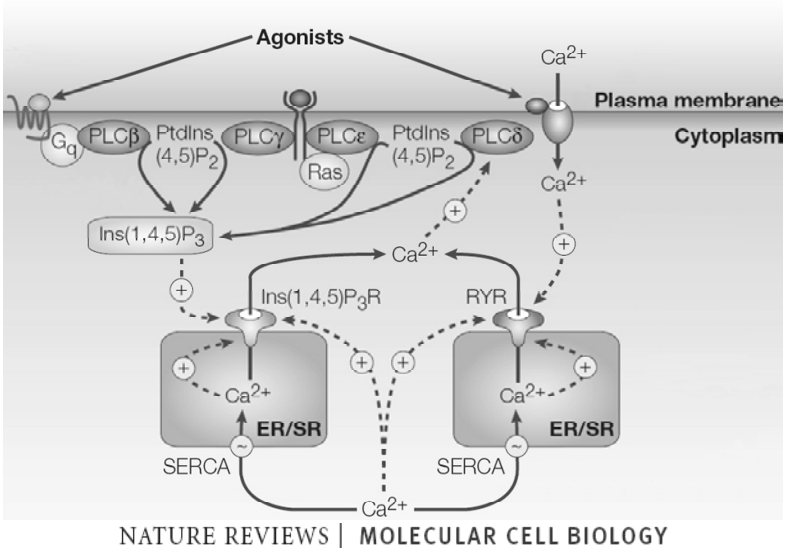

Fig. 1.6 Calcium-mobilizing messengers.

Adapted by permission from Macmillan Publishers Ltd: Nature Reviews: Molecular Cell Biology, Berridge M.J., Bootman M.D and Roderick H.L [22]. The release of Ca^{2+} from internal stores is mediated by inositol-1,4,5-trisphosphate receptor ($Ins(1,4,5)P_3R$) or the ryanodine receptor (RYR). The $Ins(1,4,5)P_3R$ is regulated by inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which is generated by various signaling pathways using different isoforms of phospholipase C (PLC; β, δ, ε, and γ). Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump increases the luminal level of Ca^{2+}, which results in sensitization of the RYR. $PtdIns(4,5)P_2$ = phosphatidylinositol-4,5-bisphosphate.

Release of Ca^{2+} from intracellular stores can increase $[Ca^{2+}]_i$ only to a limited level, which depends on the amount of Ca^{2+} stored within the ER [24]. In immune cells, the decrease of ER $[Ca^{2+}]$ triggers activation of the pathway, resulting in opening of store-operated calcium channels in the plasma membrane. It has been shown that store depletion is associated with

the oligomerization of the ER transmembrane protein STIM1, which then moves to the ER–plasma membrane junctions within 10–25 nm of the plasma membrane [27]. It is currently proposed that STIM1 directly or indirectly activates the membrane protein ORAI1, which is a subunit of a store-operated channel [28]. The functional relevance of this pathway has been recently shown in macrophages [29].

1.5.2 Macrophage immune functions dependent on calcium

In macrophages, changes in $[Ca^{2+}]_i$ have been associated with multiple functions, including, but not limited to activation, phagocytosis, microbial killing, inflammatory gene expression, and secretion.

Immune recognition by macrophages depends on the ability to sense non-self or altered-self which leads to cell activation and increased motility. Several mechanisms and positive-feedback loops establish and amplify phosphatidylinositol-3,4,5-trisphosphate signals that promote the generation of the leading edge, thus enable directional cell movement [30]. The requirement for Ca^{2+} signals in macrophage motility was recently refined by Evans and Falke [31]. Using fluorescent proteins to determine the cellular localizations of phosphoinositide 3-kinase (PI3K) activity, actin, and protein kinase C, the study reports that an extracellular Ca^{2+} influx participates in a positive-feedback cycle between PI3K and F-actin at the leading edge [31]. Moreover, Ca^{2+} localizes protein kinase C to the ruffling leading edge and supports prolonged stimulation of PI3K by chemoattractants [31].

The phagocytosis is initiated by the recognition of the particle by receptors on the macrophage surface followed by F-actin mobilization around nascent phagosomes. As the phagosome maturates, the actin rim is removed [32]. While cytoskeleton dynamics have been shown to be regulated by Ca^{2+} [33], the role of this ion in phagocytosis by macrophages was questionable. Experiments done on macrophage cell lines and elicited peritoneal macrophages (PM) using different strategies of Ca^{2+} chelation showed varying results for the requirement of Ca^{2+} for phagocytosis of opsonized and non-opsonized targets [34-38]. Experimental approaches of depleting Ca^{2+} may not be optimal for studying the necessity of Ca^{2+} in the regulation of phagocytosis. Recent studies using genetically modified mice deficient for STIM1 show impaired phagocytosis in PM, which clearly indicates the requirement of Ca^{2+} in regulation of this process [29].

Part of the killing mechanism employed by phagocytes on phagocytosed microbes depends on the generation of the superoxide anion in a metabolic process known as the respiratory burst

[14, 39]. This process depends on the assembly and activation of a multisubunit complex known as the NADPH oxidase, which has been shown to be Ca^{2+}-dependent [39, 40].

Immune recognition induces changes in gene expression that are essential for the coordination of the immune response. The transcription factor nuclear factor κB (NF- κB) is an important integrator of inflammatory response. It has been shown that in PM treated with LPS, protein-tyrosine kinases mediate PLCγ phosphorylation, which is followed by a $[Ca^{2+}]_i$ increase [41]. Several Ca^{2+}-dependent protein kinases are activated, and PKCβ regulates phosphorylation of MEKK1 protein, which then activates inhibitory κB kinase. Sequentially, NF-κB is activated, and transcription of inflammatory effector genes (inducible nitric-oxide synthase and TNFα) is promoted [41].

Secretions of AM are crucial for initiation of inflammatory response in the alveolus. Macrophages activated by LPS release TNFα by exocytosis which is accompanied by dynamic changes in proteins regulating this process [42]. A study by Di *et al.* [43] suggests that exocytosis in macrophages is triggered by an interplay between $[Ca^{2+}]_i$ and activated G-proteins. In this model, increase in $[Ca^{2+}]_i$ promotes docking of secretory vesicles to the plasma membrane, which allows G-protein-dependent vesicle fusion [43]. The role of $[Ca^{2+}]_i$ in the release of CCL2 was also studied in the rat AM NR8383 cell line. Release of this chemokine was increased after stimulation with UTP nucleotide which is known to bind to $P2Y_2$ receptors and activate Ca^{2+} transients [44]. Moreover, chelation of intracellular Ca^{2+} by BAPTA, or pharmacological inhibition of PLC abolished UTP-induced facilitation of CCL2 release [45].

1.6 *The nACh receptors in macrophages*

1.6.1 Acetylcholine synthesis, transport and degradation

Acetylcholine (ACh), a classical transmitter of parasympathetic nerve fibres in the airways, is also synthesized by a large number of non-neuronal cells [46]. Phylogenetically, ACh is much older than nervous system, as cholinergic systems are present in bacteria and primitive plants [47]. Synthesis of ACh is catalyzed by choline acetyltransferase (ChAT) or carnitine acetyltransferase (CarAT) from the substrates choline and acetyl-CoA [46]. Both substances are present in all cells of the body, since choline is important for synthesis of plasma membrane lipids and acetyl-CoA is an intermediate between glycolysis and Krebs cycle. In some cells, ACh is packed into vesicles by the vesicular acetylcholine transporter (VAChT) and released by exocytosis. However, the majority of non-neuronal cholinergic cells are not

equipped with VAChT. It has been shown, that more widely expressed polyspecific organic cation transporters mediate release of ACh [48, 49]. Once released, ACh can activate acetylcholine receptors or will be cleaved by esterases e.g. acetylcholine esterase, which is present in neuronal synapses and on the surface of red blood cells [50]. Less efficient enzymes like burtyrylcholine esterase, are present in many tissues where they also contribute to the clearance of ACh [50].

1.6.2 Nicotinic acetylcholine receptors

Receptors for acetylcholine are grouped in two large families: metabotropic muscarinic acetylcholine receptors and ionotropic nicotinic acetylcholine receptors (nAChR). This thesis will focus on the properties and functions of nAChR. As it has been outlined in section 1.4.1, nAChR are composed from 5 subunits. There are 9 different known ligand-binding α-subunits (αl-α7 and α9-α10; the α8-subunit has only been found in chicken) that assemble to homo- or heteropentamers, partially with additional participation of two copies of a β-subunit. Four different isoforms of β-subunits have been described [51]. Receptors with different subunit composition are distinct from each other with respect to ligand affinity - e.g. choline is more effective at α7-homopentamers than ACh - and to preference for mono- or divalent cations [51, 52]. Interestingly, nAChR in immune cells seem to have distinct properties from their neuronal counterparts. Binding of agonists or competitive antagonists affects the activity of adjacent (purinergic or antigen-specific) receptors and influences the $[Ca^{2+}]_i$ fluxes evoked by their stimulation [53-55]. Membrane bound nAChR subunits have been demonstrated to interact with and to modulate signaling by β-arrestin [56], phosphatidyl-inositol-3-kinase [57], CD3ζ [54], and purinergic P2X-receptors [58, 59].

1.6.3 Expression and function of nAChR in macrophages

Studies on the expression of nicotinic receptors have been hampered by the fact that antibodies to nAChR are prone to unspecific binding [60]. Because of the difficulties in the interpretation of the immunolabeling data in this context, I will provide an overview of the expression of mRNA coding for nAChR subunits in macrophages.

Studies done on the murine AM cell line MH-S showed the expression of nAChR subunits α4 and β2, but the authors did not detect the α7 subunit [61]. Similar observations were made for AM isolated from FVB mice which express α3-α6, α9, α10 and β2-β4 nAChR subunits [62]. Macrophages isolated from the *lamina propia* of the gut, PM as well as the Mf4/4 spleen

macrophage cell line showed expression of α4 and β2 nAChR subunits, whereas the α7 nAChR subunit was not present in these cells [63]. In contrast, Wang *et al.* demonstrated the expression of α7 nAChR subunit mRNA in human monocyte-derived macrophages [64]. This might reflect a species difference since a low level of basal expression of α7 nAChR subunit mRNA in AM isolated from healthy volunteers and an increase in AM isolated from smokers has been reported [65].

The functional role of nAChR in macrophages has been mostly studied in the context of the cholinergic anti-inflammatory pathway, which will be described in detail in the next section. Here, I will review aspects of macrophage biology which have been shown to be modulated by nAChR.

A recent study by van der Zanden *et al.* [63] examined the role of nAChR in the regulation of phagocytosis in PM. The phagocytosis of *Enterococcus faecium*, zymosan particles, opsonised red blood cells and acetylated low-density lipoprotein was increased in PM stimulated with nicotine. This nicotinic effect was inhibited by non-specific or α4β2 nAChR antagonist, but not by inhibitors selective for α7, α9/α10 nAChR [63]. The effects of nicotine on the capacity of macrophages for intracellular microbial killing were assessed in the MH-S cell line after infection with *Legionella pneumophilia* [61]. Nicotine treatment enhanced the replication of bacteria in the macrophages and selectively down-regulated the production of IL-6, IL-12 and TNFα, but not of IL-10, induced by infection [61]. Again, effects of nicotine were blocked by a nonselective nAChR antagonist, *d*-tubocurarine, but not by the α7, α9/α10 nAChR antagonist α-bungarotoxin [61].

1.6.4 “Cholinergic anti-inflammatory pathway”

The concept of the “Cholinergic anti-inflammatory pathway” arose from the observation that electrical stimulation of the peripheral vagus nerve decreased TNFα production during endotoxaemia in rats [66]. In endotoxaemic animals vagotomy increased serum and liver TNFα levels; this was inhibited when the transected vagus nerve was electrically stimulated. It was further shown that ACh and nicotine attenuated the release of pro-inflammatory cytokines from human monocyte-derived macrophages stimulated with LPS [66]. A pharmacological approach identified the α7 nAChR as an essential regulator of these anti-inflammatory effects in monocyte-derived macrophages [64]. This notion was supported by analysis of α7 nAChR-deficient mice in which the protective effect of vagus nerve stimulation in LPS-induced endotoxaemia was lost [64]. The anti-inflammatory effects of nicotine were shown to be dependent on the activation of the transcription factor STAT3. A study by de

Jonge *et al.* [67] suggested that in mouse PM STAT3 was phosphorylated by the tyrosine kinase JAK2, which was recruited to the α7 nAChR. Surprisingly, a recent publication from this group shows that mouse PM do not express α7 nAChR mRNA [63]. However, α-bungarotoxin and methyllycaconitine (antagonists at nAChR containing homo- and heteromeric α-subunits, including α7 and α9 subunits) or dihydro-β-erythroidine (antagonists at nAChR composed of α4β2 subunits) abrogated protective effects of nicotine on TNFα production in zymosan-induced PM [63]. No evidence for the expression of α7 nAChR in monocytes/macrophages has also been obtained in earlier studies by other groups [55, 61, 62]. While the general role of α7 nAChR as a modulator of inflammation it well established by experiments in α7 subunit-deficient mice [64, 68, 69], the actual localization of the cholinergic synapse which mediates the protective effects is elusive. Of note, the laboratory of Kevin Tracy [64] was not the first to identify the interaction between nicotine acting through α7 nAChR and TNFα. Numerous tissue culture studies have identified interactions between these pathways that impact neuroprotection to neurons challenged with excitotoxins [70-72].

1.7 *The role of 5-HT in macrophage biology*

1.7.1 Synthesis and regulated release of 5-HT

Serotonin (5-hydroxytryptamine, 5-HT) is well known for its role in vasoconstriction as well as in diverse biological processes such as tissue regeneration [73], platelet activation [74], and immune system regulation [75]. Outside the central nervous system, most of the bodies' 5-HT is produced by enterochromaffin cells. The synthesis of 5-HT from trytophan requires two enzymatic reactions. The first, rate limiting step is catalyzed by tryptophan hydroxylase (TPH) [76]. 5-Hydroxy-L-tryptophan is then converted to 5-HT by the action of the widespread aromatic L-amino acid decarboxylase. Two isoforms of TPH have been described: TPH1 is expressed in some peripheral tissues, including duodenum, while TPH2 expression is limited to the brain [76]. Serotonin produced in the gut is released upon stimulation into the circulation, where it is taken up by and stored in platelets which express the serotonin reuptake transporter (SERT). Platelets release 5-HT upon activation in response to vascular wall injury, LPS [77] or allergen challenge [78]. Together with 5-HT released from mast cells [79], this results in increased 5-HT levels in inflammation [75]. In addition, activated T-cells [80] and macrophages [81] express TPH1, and dendritic cells [80], monocytes [82] and macrophages [83] express SERT which translocates 5-HT across the plasma membrane.

1.7.2 5-HT receptors

Serotonin receptors build up a large family including ionotropic cation channels (5-HT_3) and six types of metabotropic G-protein coupled receptors (Tab. 1.2) [84].

Receptor type	Signal transduction pathway	Mechanism of action
5-$HT_{1A, B, D, E, F}$	$G\alpha_i/G\alpha_O$ protein coupled	↓ cAMP
5-$HT_{2A, B, C}$	$G\alpha_q/G\alpha_{11}$-protein coupled	↑ Ins(1,4,5)P_3 and DAG
5-$HT_{3A, B}$	Ligand-gated Na^+ and K^+ ion channel	Depolarization of plasma membrane
5-HT_4	$G\alpha_s$-protein coupled	↑ cAMP
5-HT_{5A}	$G\alpha_i/G\alpha_o$-protein coupled	↓ cAMP
5-HT_6	$G\alpha_s$-protein coupled	↑ cAMP
5-HT_7	$G\alpha_s$-protein coupled	↑ cAMP

Tab. 1.2 Classification of 5-HT receptors

Receptors are classified by operational, structural and transductional criteria. ↑ denotes increase, ↓ decrease in cellular levels of second messenger. cAMP = cyclic adenosine monophosphate, Ins(1,4,5)P_3 = inositol-1,4,5-trisphosphate, DAG = diacylglycerol

Peripheral blood mononuclear leukocytes, B-cells, T-cells, and dendritic cells express various subtypes of metabotropic 5-HT receptors, which have been shown to modulate cell activation, proliferation and cytokine and chemokine production [75, 80, 85-87]. Less is known about the expression and function of 5-HT receptors on macrophages. Early experiments with bone-marrow derived murine macrophages identified 5-HT as the modulator of induced MHC class I and II expression. However, effects were inconsistent in different studies [75]. Conversely, innate immunity functions (IFNγ-induced phagocytosis, superoxide production) are generally enhanced by 5-HT [88, 89]. More recently, 5-HT and 5-HT_{1A} receptor-specific agonists were shown to increase phagocytosis of zymosan particles by PM [90].

A serotonergic modulation of cytokine secretion by NR8383 cells and human AM has been reported. Low doses of 5-HT (10^{-10}–10^{-9} M) decreased TNFα and stimulated IL-10 production in both control and LPS-treated NR8383 cells [91]. It has been suggested that 5-HT_2 receptors are involved in the modulation of cytokine secretion, because the 5-HT_2 selective agonist DOI reproduced the effect of 5-HT [91]. However, use of the 5-HT_2 receptor antagonist ritanserin,

did not abrogate the inhibitory effect of 5-HT on AM TNFα release, suggesting the participation of other 5-HT receptors [91].

1.8 *Aim of the study*

Alveolar macrophages, the major immune cells in the lung, express a multitude of receptors which allow them to sense their environment and orchestrate immune response by secreting cytokines and chemokines. Their activity is controlled in a paracrine fashion by soluble mediators released from surrounding cells. Activation of AM involves generation of Ca^{2+} signals which direct cells to perform specific immune functions.

The present study was aimed to determine to which extend, and via which receptor, 5-HT, an amine released from activated platelets, and nAChR, a receptor class that has been identified in mediating anti-inflammatory effects, also participate in paracrine modulation of AM function. To this end the expression of nAChR and 5-HT receptors in AM was examined and functional changes in $[Ca^{2+}]_i$ in response to paracrine signals were monitored. The role of 5-HT in modulation of AM was further explored by studying chemokine and cytokine secretions.

2 Materials and methods

2.1 *Materials*

2.1.1 Animals

Female Wistar rats were obtained from the local animal breeding facility (Institute of Physiology, Justus-Liebig-University, Giessen, Germany).

Male and female wild-type C57BL6N mice were purchased from Charles River (Sulzfeld, Germany). Wild-type Swiss Webster mice were purchased from Charles River (Portage, USA). All animals were kept in specific-pathogen free facility with free access to food and water. Mice were used throughout the study between 8 and 12 weeks of age.

The animals deficient for 5-HT_{2C} receptor were generated by Dr. Laurence H. Tecott (Department of Psychiatry, University of California, San Francisco, USA) [92] and obtained from Dr. Pete Clifton (Department of Psychology, University of Sussex, Falmer, Brighton, UK). Mice were bred using heterozygotes as breeders by Mfd Diagnostics (Wendelsheim, Germany). Offspring was genotyped using primer pairs for the 5-HT_{2C} gene and the neomycin resistance cassette. Mice were used throughout the study between 8 and 12 weeks of age. All animals were kept with free access to food and water under conventional housing conditions. Experimental protocols involving animals were approved by institutional and local government comities, following the current version of the Law on the Protection of Animals as well as the NIH “principles of laboratory animal care”.

2.1.2 Equipment

Calcium imaging system	TILL Photonics, Germany
Cell culture incubator	Heraeus, Germany
Confocal Laser Scanning Microscope, TCS SP2	Leica, Germany
Cryostat, HM560 Microstar	Microm, Germany
Electrophoresis apparatus	PeqLab, Germany
Eppendorf tubes (1.5 ml/2 ml)	Eppendorf, Germany
Filter tip	Greiner bio-one, Germany
Filter units	Millipore, USA
Fluorescence 8 chamber glass slides	BD, Germany
Fluorescence microscope, Axioplan 2	Zeiss, Germany
Flow cytometer, FACSCalibur	BD, USA

Gel documentation system	Phase, Germany
MasterCycler Personal	Eppendorf, Germany
MasterCycler Gradient	Eppendorf, Germany
Microscope, BX50W	Olympus, Germany
Mini spin centrifuge, Biofuge Fresco	Heraeus, Germany
Mini Trans Blot	Hoefer, USA
Mixer Mill, MM 301	Retsch, Germany
Multifuge centrifuge	Heraeus, Germany
PCR tubes (0.2 ml)	Applied Biosystems, USA
Pipetmans: P10, P20, P100, P200, P1000	Gilson, France
Pipette tip	BD, Germany
Power supply	Hoefer, USA
Safety Cabinet, HERAsafe	Heraeus, Germany
Serological pipette: 5, 10, 25, 50 ml	BD Falcon, Germany
Spectrophotometer, Biophotometer	Eppendorf, Germany
Test tubes: 15, 50 ml	Greiner bio-one, Germany
Test tube thermostat	Kleinfeld, Germany
Tissue culture plates: 24 well	Greiner bio-one, Germany

2.1.3 Kits

DNeasy extraction kit	Qiagen, Germany
iScript	Bio-Rad, Germany
Mouse Cytokine Antibody Array, Panel A (ARY006)	R&D Systems, Germany
RNeasy Mini kit	Qiagen, Germany
SuperScript II	Invitrogen, Germany
SuperSignal West Pico ECL detection kit	Thermo, USA

2.1.4 Reagents

Acetic Acid	Merck, Germany
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Germany
1-chloro-2,2,2-trifluoroethly difluoromethyl ether (Isoflurane)	Abbott, Germany

Acetone	Merck, Germany
Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Peqlab, Germany
Ammonium persulfate (APS)	Promega, Germany
AmpliTaq DNA polymerase	Applied Biosystems, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany
Bromophenol blue	Sigma-Aldrich, Germany
Calcium chloride ($CaCl_2$•$2H_20$)	Merck, Germany
D-glucose	Sigma-Aldrich, Germany
Dithiothreitol (DTT)	Invitrogen, Germany
DMEM/F12 GlutaMax-I medium	Invitrogen, Germany
DNA low range marker (100 bp)	Fermentas, USA
dNTPs	Applied Biosystems, USA
Dublecco's phosphate buffered saline (PBS)	PAA Laboratories, Austria
Ethanol	Carl Roth, Germany
Ethidium bromide solution	Carl Roth, Germany
Ethylendinitrilo-N, N, N´, N´, -tetra-acetic acid disodium salt (EDTA)	Fluka, Germany
Ethylene glycol-bis (2-amino-ethylether)-N,N,N',N'–tetra-acetic-acid (EGTA)	Sigma-Aldrich, Germany
Glycerol	Merck, Germany
Glycine	Sigma-Aldrich, Germany
Hepes	Sigma-Aldrich, Germany
Hydrochloric acid (HCl)	Merck, Germany
Isopropanol	Merck, Germany
Magnesium chloride ($MgCl_2$, 15 mM) solution	Applied Biosystems, USA
Magnesium chloride ($MgCl_2$•$6H_2O$)	Merck, Germany
β-mercaptoethanol	Sigma-Aldrich, Germany
Methanol	Fluka, Germany
N,N,N',N'-tetramethyl-ethane-1.2-diamine (TEMED)	Sigma-Aldrich, Germany
Paraformaldehyde	Merck, Germany
PCR buffer II	Applied Biosystems, USA
Penicillin-streptomycin	PAA Laboratories, Austria

Polyvinylidene difluoride (PVDF) membranes	Bio-Rad, USA
Potassium chloride (KCl)	Merck, Germany
Precision Plus Protein^{TM} Standards	Bio-Rad, USA
Protease inhibitor cocktail	Roche, Germany
Random hexamers	Boehringer, Germany
Sodium chloride (NaCl)	Roth, Germany
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Germany
Sodium hydroxide (NaOH)	Merck, Germany
Tissue Tek O.C.T compound	Sakura, Netherlands
Tris	Carl Roth, Germany
Tween 20	Sigma-Aldrich, Germany

2.2 *Methods*

2.2.1 Genotyping

Mouse tail biopsies were obtained and DNA was prepared using the DNeasy extraction kit (Qiagen, Germany) according to manufacturer's instructions. Genotyping was performed with the following primer sets detecting wild type 5'-AGT TGA TGT TCA TCT CAG GTG GC-3' (fwd), 5'-GGG TCC TAT AGA TCG AGG TAC C-3' (rev) and mutated 5-HT_{2C} gene locus 5'-CAC CTT GCT CCT GCC GAG AAA-3' (fwd), 5'-AGA AGG CGA TAG AAG GCG ATG-3' (rev). PCR amplification was carried out as describe in details below (see section 2.2.7), except that cycling conditions were 10 min at 95°C, 35 cycles with 30 s at 94°C, 30 s at 57°C, 20 s at 72°C, and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on a 1.5% agarose gel in Tris-acetate-EDTA buffer (Fig. 2.1). Negative controls were run by adding H_2O instead of the DNA template.

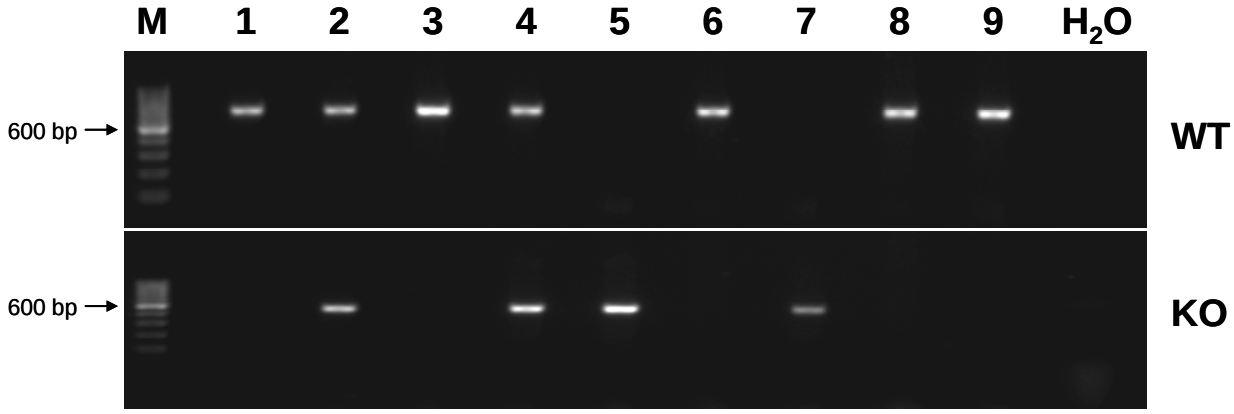

Fig. 2.1 PCR genotyping of the 5-HT_{2C} receptor-deficient mice.
DNA extracted from tail snips was analyzed by PCR with primers detecting wild type (upper panel) and mutated version (lower panel) of 5-HT_{2C} gene. Lanes 1-9 show results for individual mice. Animals 1, 3, 6, 8, and 9 are carrying the wild type allele. Mice 5 and 7 are 5-HT_{2C}-deficient, while animals 2 and 4 are heterozygous. H_2O = negative control, M = DNA marker

2.2.2 Isolation of rat alveolar macrophages

Animals were killed by inhalation of an overdose of isoflurane (Abbott, Germany). The lung was carefully removed, cannulated via the trachea, and bronchoalveolar lavage (BAL) was performed using 10 x 5 ml ice-cold PBS (PAA, Austria) containing (in mM): KCl 2.68, KH_2PO_4 1.47, NaCl 136.89, Na_2HPO_4 8.10 (pH 7.3). The lavage fluid was centrifuged at 400 g for 5 min at 4°C, and the pellet was resuspended in PBS or DMEM/F12 GlutaMax-I medium (Invitrogen, Germany) supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml). BAL cells were monitored by microscopy, and preparations containing erythrocytes were discarded. Cells were purified by adherence for at least 1 h and washed.

2.2.3 Isolation of mouse alveolar macrophages

BAL fluid was obtained by cannulating the trachea with a shortened 21 G needle attached to a 1-ml insulin syringe, followed by repeated intratracheal instillations with 500 µl aliquots of sterile PBS/2 mM EDTA (pH 7.2) until a BAL fluid volume of 5 ml was recovered. The lavage fluid was centrifuged at 400 g for 10 min at 4°C, and the pellet was resuspended in PBS, RPMI 1640 or DMEM/F12 GlutaMax-I medium (Invitrogen) supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were purified by adherence for at least 30 min and washed.

2.2.4 NR8383 cell culture

The rat NR8383 AM cell line (CRL-2192, ATCC, LGC Standards, UK), was cultured in DMEM/F12 medium supplemented with 15% heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) at 37°C and 5% CO_2. Before the experiments, cells were centrifuged at 400 g for 10 min at 22°C, and the pellet was resuspended in DMEM/F12 medium.

2.2.5 Flow cytometric analysis

Cells obtained by BAL were resuspended in PBS/1% BSA and counted using a Neubauer chamber. Cells were incubated with directly conjugated monoclonal antibodies (eBiosciences, USA), which are listed in Tab. 2.1. Controls were incubated with isotype-matched control antibodies.

Antibody	Clone	Isotype	Label	Amount used for 1 staining [µg]
CD45	30-F11	Rat IgG2b, κ	FITC	0.125
CD11b	M1/70	Rat IgG2b, κ	APC	0.05
CD11c	N418	Armenian Hamster IgG	PE	0.03
Gr-1	RB6-8C5	Rat IgG2b, κ	PE	0.05

Tab. 2.1 Antibodies used for flow cytometry on mouse cells.
FITC = Fluorescein isothiocyanate, APC = Allophycocyanin, PE = Phycoerythrin

Staining was performed at 4°C in the dark for 30 min. After staining, cells were washed twice in PBS/1% BSA. Samples were acquired using a FACSCalibur flow cytometer equipped with an argon-ion laser at 488 nm excitation wavelength and a red-diode laser at 633 nm excitation wavelength and CellQuest software (BD, USA), and the data were analyzed with FlowJo software (Tree Star, USA).

2.2.6 RNA isolation and cDNA synthesis

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany). RNA was isolated following the instructions of the manufacturer and was quantified spectrophotometrically using a Biophotometer (Eppendorf, Germany).
Genomic DNA contaminations were removed by DNAse I (Invitrogen) digestion for 15 min at 37ºC, 1 U/reaction. Synthesis of cDNA was performed with iScript (Bio-Rad, Germany) or SuperScript II (Invitrogen) kits using 1 µg total RNA. For the iScript cDNA synthesis, the following reagents were combined:

Component	Amount per reaction
5x iScript reaction mix	4 µg
iScript reverse transcriptase	1 µl
RNA	1 µg
H_2O	up to 20 µl

Conditions for the reaction were 5 min at 25ºC, 30 min at 42ºC, and 5 min at 85ºC.

Alternatively, mRNA was reversely transcribed using SuperScript II kit. For the reaction, the following reagents were combined:

Component	Amount per reaction
mRNA	1 µg
Oligo d(T) (500 µg/ml)	1 µl
dNTP mix (10 mM each)	1 µl
H_2O	Up to 12 µl

To anneal oligo d(T) primers to mRNA, reagents were mixed, heated to 65ºC for 5 min, and quickly chilled on ice. Then, 5X First-Strand Buffer (4 µl) and 0.1 M DTT (2 µl) were added to the tube. Components were mixed and incubated 2 min at 42ºC. Superscript II reverse transcriptase was added (1 µl) and reagents were mixed by gentle pipeting. The tube was incubated at 42°C for 50 min. The reaction was terminated by heating up the components to 70°C for 15 min. After synthesis, cDNA was stored in -20ºC.

2.2.7 RT-PCR

The cDNAs were amplified with the subunit specific primer pairs spanning at least one intron (for primers detecting rat sequences, see Tab. 2.2 mouse primers are listed in Tab. 2.3).

Target	Sequence		Length	Gene Bank Accession No.
nAChR α1	for.	AGCTCACCGCTGTCCTCCT	171 bp	NM_024485
	rev.	GGATCAGTTGCAGTCCCACA		
nAChR α2	for.	CGCGTCCCTTCAGAGATGAT	114 bp	L31622
	rev.	CACAGTGCCCGTGAAGAA		
nAChR α3	for.	CCTCCCTGTCTATCGGGTCT	161 bp	X03440
	rev.	GCCGGATGATCTCGTTGTAA		
nAChR α4	for.	GGACCCTGGTGACTACGAGA	137 bp	NM_024354
	rev.	CATAGAACAGGTGGGCCTTG		
nAChR α5	for.	TGGAACACCTGAGCGACAAG	284 bp	NM_017078
	rev.	CGTGACAGTGCCGTTGTACC		
nAChR α6	for.	TGGTGTTAAGGACCCCAAAA	142 bp	NM_057184
	rev.	GCTGCTGGCTTAACCTCTTG		
nAChR α7	for.	ACATTGACGTTCGCTGGTTC	235 bp	L31619
	rev.	CTACGGCGCATGGTTACTGT		
nAChR α9	for.	CGTGGGATCGAGACCAGTAT	142 bp	AY574257
	rev.	TCATATCGCAGCACCACATT		
nAChR α10	for.	GTGCCACTCATCGGAAAGTA	107 bp	NM_022639
	rev.	TGTGCATTAGGGCCACAGTA		
nAChR β1	for.	TCCTAAGCGTGGTGGTCCTC	151 bp	NM_01258
	rev.	TGTGGTTCGGGTAGTTGGTC		
nAChR β2	for.	AGCCTTCTTTGGCTGTGCTC	116 bp	NM_019297
	rev.	GAGCCGTTAGTAGCTGGACGA		
nAChR β3	for.	CACTCTGCGCTTGAAAGGAA	196 bp	NM_133597
	rev.	GCGGACCCATTTCTGGTAAC		
nAChR β4	for.	CACTCGCGGTTCCATTGTAG	159 bp	NM_052806
	rev.	CGGGTTTTGTTCAGGAGGTC		
P2Y1	for.	AGGAAAGCTTCCAGGAGGAG	203 bp	NM_012800
	rev.	GGCCAATAGAATGTTGCTTCTT		
P2Y2	for.	CATGCGAGTGAAGAACTGGA	209 bp	NM_017255
	rev.	GGCAGCAGCACATACTTGAA		
P2Y4	for.	AGTCCCTGGGCTGGACTAAG	267 bp	NM_031680
	rev.	GTGTCTGACAATGCCAGGTG		
HPRT1	for.	TCCCAGCGTCGTGATTAGTG	225 bp	NM_012583
	rev.	TCCAGCAGGTCAGCAAAGAA		

Tab. 2.2 Rat primer sequences used in the study.

Sequences for forward (for.) and reverse (rev.) primers are given in 5`→3` order.

Target	Sequence		Length	Gene Bank Accession No.
$5HT_{2A}$	for	ATAGCCGCTTCAACTCCAGA	106 bp	NM_172812.1
	rev	TCATCCTGTAGCCCGAAGAC		
$5HT_{2B}$	for	GGGCTACTGCATTCATCAAGA	119 bp	NM_008311.2
	rev	CTCACAGGTGACATTGTGTGG		
$5HT_{2C}$	for	GTTCAATTCGCGGACTAAGG	116 bp	NM_008312.4
	rev	TCACGAACACTTTGCTTTCG		
nAChR α7	for	ACAATACTTCGCCAGCACCA	144 bp	AF225980
	rev	AAACCATGCACACCAGTTCA		
nAChR α9	for	CAATGCTCTGCGTCCAGTAG	209 bp	XM_132045
	rev	ACACCAGATCGCTGGGAATC		
nAChR α10	for	TCTGCTCCTGCTCTTTCTCC	208 bp	XM_89067
	rev	CCACAGGTACAAGGTCAGCA		
nAChR β2	for	GAGTGTGAGGGAGGATTGGA	139 bp	AY574268
	rev	TCGTGGCAGTGTAGTTCTGG		
nAChR β4	for	CAGCCCATCCAACCTCTATG	156 bp	NM_148944
	rev	CTGACGCCCTCTAATGCTTC		
β-MG	for	ACCCTGGTCTTTCTGGTGCT	150 bp	NM_009735
	rev	AATGTGAGGCGGGTGGAA		

Tab. 2.3 Mouse primer sequences used in the study.

Sequences for forward (for.) and reverse (rev.) primers are given in 5`→3` order.

Hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) or beta microglobulin (β-MG) primers were used to monitor cDNA integrity. For one reaction, the following reagents were combined:

Component	Volume per reaction
PCR buffer II (100 mM Tris-HCl, 500 mM KCl, pH 8.3)	2.5 µl
$MgCl_2$ (15 mM)	2 µl
dNTP (10 mM each)	0.6 µl
forward and reverse primer (10 µM each)	0.6 µl
ApliTaq Gold polymerase (5 U/µl)	0.125 µl
DNA or cDNA	1 µl
H_2O	up to 25 µl

Cycling conditions were 10 min at 95°C, 35 cycles with 30 s at 94°C, 30 s at 57-60°C, 20 s at 72°C, and a final extension at 72°C for 10 min.

PCR products were separated by electrophoresis on gels prepared with 1x Tris-acetate-EDTA buffer + 1.5 % agarose (Peqlab, Germany) + 0.5 mg/ml ethidium bromide. Tris-acetate-EDTA buffer was prepared as a 50x Tris-acetate-EDTA stock (pH 8.0). For 50x Tris-acetate-EDTA, 242 g Tris base, 75.1 ml glacial acetic acid, and 37.2 g Na_2EDTA were dissolved in H_2O and the volume was adjusted to 1000 ml with H_2O.

Electrophoresis was run at a constant voltage of 150 V for 30 min. PCR products were visualized under UV light and documented using a gel documentation system (Phase, Germany). An 100 bp DNA marker (Fermentas, USA) served as marker for PCR product size. Positive controls for primers were run by using reversely transcribed total RNA from tissues abundantly expressing the respective target sequences (Tab. 2.2).

Target	Positive control
nAChR subunits α2-α10	DRG[a], brain[b]
nAChR subunits α1, β1	Skeletal muscle
nAChR subunits β2-β4	Lung
$P2Y_{1,2,4,6}$ receptors	Spleen
$5\text{-}HT_{2A,B,C}$ receptors	Brain

Tab. 2.4 Tissues used as positive controls for RT-PCR reaction with target-specific primers

[a] for primers specific to rat sequences [b] for primers specific to mouse sequences.

Synthesis of primers and sequencing of PCR products was done by MWG Biotech (Ebersbach, Germany). Negative controls were performed by omitting the reverse transcription step or by using H_2O instead of cDNA template.

2.2.8 Immunofluorescence

Lavaged cells were plated on polystyrene 8-well culture slides (BD Biosciences, Belgium) in DMEM/F12 supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were allowed to attach for 2 h, fixed in acetone (-20°C, 10 min) or isopropanol (4°C, 10 min) and air-dried for 1 h.

Shock-frozen lung specimens were prepared by immersion in freezing isopentane with O.C.T. compound (Sakura, Netherlands) cryoprotection. Cryostat sections were cut at 10 µm thickness, fixed as above and subjected to indirect immunofluorescence using antisera

directed against nAChR subunits and monoclonal antibody ED1 [93], directed to a CD68-like antigen expressed by AM (Serotec, Germany). For the detection of the 5-HT_{2C} receptor in mouse lung cryostat sections, a goat polyclonal antibody (sc-15081, Santa Cruz, USA) was used. Detailed information on the antibodies is provided in Tab. 2.5.

Target	Immunogen	Host	Dilution	Source
nAChR α3	Synthetic peptide (aa 466-474 of human sequence)[a]	Rabbit	1:1600	Acris
nAChR α4	Synthetic peptide (620-627, human) [a]	Rabbit	1:800	Acris
nAChR α5	Synthetic peptide (460-468, human) [a]	Rabbit	1:1600	Acris
nAChR α7	Synthetic peptide (493-502, human) [a]	Rabbit	1:1000	Acris
nAChR α7	Native and denatured α7 subunit (380-400, chicken) and denatured α7 subunit from rat	Mouse, monoclonal, clone mAb 306	1:750	Sigma-Aldrich
nAChR α9	Synthetic peptides (81-97 and 115-128, rat)	Guinea-pig	1:1000	Kurzen *et. al.* [94]
nAChR α10	Synthetic peptide (404-418, rat) [a]	Rabbit	1:2000	Own, Lips *et. al.* [95]
	Synthetic peptide (404-418, rat) [a]	Guinea-pig	1:4000	
nAChR β2	Synthetic peptide (493-502, human) [a]	Rabbit	1:1600	Acris
nAChR β3	Synthetic peptide (450-458, human) [a]	Rabbit	1:800	Acris
nAChR β4	Synthetic peptide (490-498, human) [a]	Rabbit	1:3200	Acris
CD68-like	Rat spleen cells	Mouse, monoclonal, clone ED1	1:800	Serotec
sc-15081	Syntetic peptide (mapping N-terminus, human)[a]	Goat	1:100	Santa Cruz

Tab. 2.5 Primary antibodies used in the study.

Origin and regions of nAChR subunits and 5-HT_{2C} receptor sequences used for generation of the antibodies are provided in brackets. [a]Peptides available for preabsorption.

Unspecific binding sites were saturated with 50% normal horse serum in PBS for 1 h, followed by overnight incubation with the primary antibody, washing (3 x 10 min) and application of the secondary antibody (Tab. 2.4) for 1 h. In some preparations, cell nuclei were stained with DAPI (Sigma-Aldrich) (10 min, 1 µg/ml). Slides were washed, fixed in buffered 4% paraformaldehyde, coverslipped in carbonate-buffered glycerol (pH 8.6) and examined with a Zeiss Axioplan 2 microscope (Zeiss, Germany) and a confocal laser scanning microscope (CLSM, TCS SP2, Leica, Germany) using argon and HeNe lasers equipped with appropriate filter sets.

Immunogen	Host	Dilution	Source	Fluorochrome	Format
rabbit Ig	donkey	1:2000	Chemicon, Germany	Cy3	IgG
guinea pig Ig	donkey	1:800	Dianova, Germany	Cy3	IgG
mouse Ig	donkey	1:400	Dianova	FITC	Ig
guinea pig Ig	donkey	1:400	Dianova	Texas Red	Ig
goat Ig	donkey	1:1000	Chemicon	Cy3	Ig

Tab. 2.6 Secondary antibodies used in the study.

Positive controls were run on shock-frozen and acetone-fixed DRG, brain or gut sections. The specificity of the immunolabeling was validated by omission of the primary antibody or preincubation with the corresponding antigen. Preabsorption was done by mixing antibodies with the peptide used for immunization (14-24 µg peptide per 100 µl of antibody solution) for 1 h before application on slides. Peptides were obtained from the same source as the antibodies (Table 2.3). Suitability of the sc-15081 antibody was tested by immunolabeling of cryostat sections of 5-HT_{2C} receptor-deficient and wild-type mice lungs.

2.2.9 SDS polyacrylamide gel electrophoresis and immunoblotting for nAChR α7 and α10 subunits detection

Snap-frozen BAL cells, rat brain and skin samples were homogenized and boiled in Laemmli's sample buffer containing:

Component	Concentration
1 M Tris-Cl pH 6.8	62.5 mM
Glycerol	10% (v/v)
β-Mercaptoethanol	4% (v/v)
Sodium dodecyl sulfate	2.3% (m/v)
Bromophenol blue	0.001% (m/v)
Complete® protease inhibitor cocktail	1 x

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was done in 1 x SDS-running buffer (90 mA for 1.5 h). Gels were composed of 5% stacking gel and 15% separating gel according to Laemmli [96].

1 x SDS running buffer

Component	Concentration
Tris base	25 mM
Glycine	250 mM
SDS	0.1% (v/v)

Stacking gel 5 %

Component	Concentration
Tris (pH 6.8)	125 mM
Acrylamid	5 %
SDS	0.1% (w/v)
Temed	0.1%(v/v)
APS	0.1% (w/v)

Separating gel 15 %

Component	Concentration
Tris (pH 8.8)	375 mM
Acrylamid	15 %
SDS	0.1% (w/v)
Temed	0.1%(v/v)
APS	0.1% (w/v)

Samples (5 x 10^4 cells) and Rainbow™ colored molecular mass markers (Amersham Pharmacia Biotech, Germany) were separated on the same gel. Proteins were transferred electrophoretically onto Immobilon™-P PVDF membranes (Millipore, USA) using a blotting buffer consisting of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol and 0.05% (w/v) SDS.

Membranes were pre-incubated with PBS containing 10% (v/v) Rotiblock (Roth, Germany) solution for 1 h. Primary mouse-anti-nAChR α7 antibodies (1:1000, clone mAb 306, Sigma-Aldrich, Germany) were diluted in blocking solution and incubated with membranes overnight at 4°C. For the detection of α10 subunits, membranes were pre-incubated with PBS containing 5% (w/v) non-fat milk powder (Roth) and guinea pig-anti-nAChR α10 (1:4000, [95]) antibodies were used. Blots were washed in PBS, 0.05% (w/v) Tween 20, and bound primary antibodies were detected by horseradish peroxidase-conjugated immunoglobulins (DAKO, Germany) in PBS, 2% low fat milk, 0.05% Tween 20 (TPBS). Secondary antisera were rabbit anti-mouse IgG (1:5000 in TPBS + 1% normal rat serum) and rabbit-anti-guinea pig IgG (1:5000 in TPBS + 2% low fat milk). Peroxidase activity was visualized by SuperSignal® West Pico Chemiluminescent Substrate (Pierce, USA) using the Kodak Scientific Imaging Film X-OMAT™ LS (Eastman Kodak, USA). Gels and blots were documented and densitometrically analyzed using a digital gel documentation system (Biozym, Germany).

2.2.10 Intracellular calcium concentration measurements

Recordings of $[Ca^{2+}]_i$ were performed after 3-8 h in primary culture (n=3 animals and 10-12 coverslips for each experimental setup). Experiments with NR8383 cells were done after 2-4 h in culture (n=3). Measurements were done in Hepes buffer.

Hepes buffer

Component	Concentration [mM]
KCl	5.6
NaCl	136.4
$MgCl_2$	1
$CaCl_2$	2.2
D-glucose	11
Hepes	10

In some experiments, $CaCl_2$ was omitted from the buffer composition and the buffer was supplemented with EGTA (3×10^{-6} M). Cells were loaded for 30 min with fura-2 AM (3.3×10^{-6} M, Invitrogen) and washed 3 x 10 min. Measurements were done in Hepes buffer at a constant temperature of at 34 °C. Fluorescence images were taken with a slow scan CCD camera system with fast monochromator (TiLL Photonics, Germany) coupled to an inverted

microscope with 20x and 40x water immersion objectives (Olympus, Germany). Fura-2 was excited at 340 and 380 nm wavelengths (λ), and fluorescence was collected at λ>420 nm. Each cell was independently tracked, and the fluorescence intensity ratio of 340/380 nm was recorded. Viability of the cells was monitored after measurements with Trypan Blue exclusion. Ratio values were normalized to 100% at the beginning of the recording. Curves were plotted from recordings done in preparations from n≥3 animals. Data are shown as mean±SEM.

2.2.11 Drugs

Drugs were dissolved in double distilled water or Hepes buffer as 10^{-2}-10^{-4} M stock solutions and stored in aliquots at -20°C. All solutions were pre-warmed to 37°C before use.

Drug	Action	Concentration [M]	Source
Adenosine 5′-triphosphate di(tris) salt dihydrate (ATP)	Agonist at P2Y, P2X receptors	10^{-4}	Sigma-Aldrich
Epibatidine	Agonist at neuronal type nAChR	10^{-6}	Sigma-Aldrich
Nicotine hydrogen tartrate	Agonist at nAChR	10^{-4}	Sigma-Aldrich
α-bungarotoxin	Antagonist at nAChR α7 and α9/α10	10^{-7}	Sigma-Aldrich
5-hydroxytryptamine (5-HT)	Agonist at 5-HTR	10^{-5}	Sigma-Aldrich
RS102221	Antagonist at 5-HT_{2C} receptor	10^{-8}	Tocris

Tab. 2.7 Drugs used in the study.

2.2.12 Dot-blot based mouse cytokine antibody array

Mouse AM were cultured in RPMI 1640 medium containing 1% BSA, L-glutamine, and penicillin/streptomycin at 5 x 10^5 cells/well in 24-well tissue culture plates. Cells were stimulated with 10^{-5} M 5-HT for 24 h. In controls, 5-HT was omitted from the media. Culture supernatants were used for qualitative measurement of cytokine expression using the Mouse Cytokine Antibody Array, Panel A (ARY006), as recommended by the manufacturer (R&D Systems, Germany).

2.2.13 Statistical analysis

Data in the figures and text are expressed as mean±SEM. Recordings of $[Ca^{2+}]_i$ were statistically analyzed by comparing the differences in Fura-2 ratio before and after addition of test substance, among different treatment groups. The non-parametric rank based Kruskal-Wallis test was used to compare multiple groups, and if significant differences were detected, it was followed by Mann-Whitney test to compare between two experimental groups. Tests were performed using SPSS software (SPSS software, Germany). $P \leq 0.05$ was considered significant and $P \leq 0.01$ as highly significant.

2.3 *Experimental protocols*

2.3.1 Rat alveolar macrophages

Cells were exposed to nicotine (10^{-6}-10^{-4} M) or epibatidine (10^{-6} M). Controls were performed with vehicle treatment. Two min after administration of nicotine or epibatidine, cells were stimulated with ATP (10^{-4} M). In some experiments, cells were exposed to nicotine in the presence of the nAChR α7 and α9/α10 blocker α-bungarotoxin (10^{-7} M). Measurements were done in buffer with or without calcium, as indicated. Cells that did not respond to ATP by an at least 5% change in $[Ca^{2+}]_i$ were excluded from further analysis.

2.3.2 Mouse alveolar macrophages

Cells were exposed to 5-HT (10^{-5} M) or ATP (10^{-4} M) alone or in the presence of nicotine, given 2 min prior to agonist. In some experiments, the extracellular buffer lacked calcium and was supplemented with EGTA (3×10^{-6} M). In an other set of experiments, AM were pre-incubated with the 5-HT_{2C} receptor blocker RS102221 compound (10^{-8} M) for 2 min and then were stimulated with 5-HT (10^{-5} M). Controls were performed with 1) vehicle treatment, and 2) ATP (10^{-4} M) serving as a positive control. Cells that did not respond to 5-HT or ATP by an at least 5% change in $[Ca^{2+}]_i$ were excluded from further analysis.

3 Results

3.1 *Expression of nAChR on freshly isolated rat alveolar macrophages*

3.1.1 Purity of isolation

To assess purity of isolation of rat AM, indirect immunofluorescence labeling was performed. The vast majority of BAL cells showed strong staining with ED1 antibody, an AM marker [93].

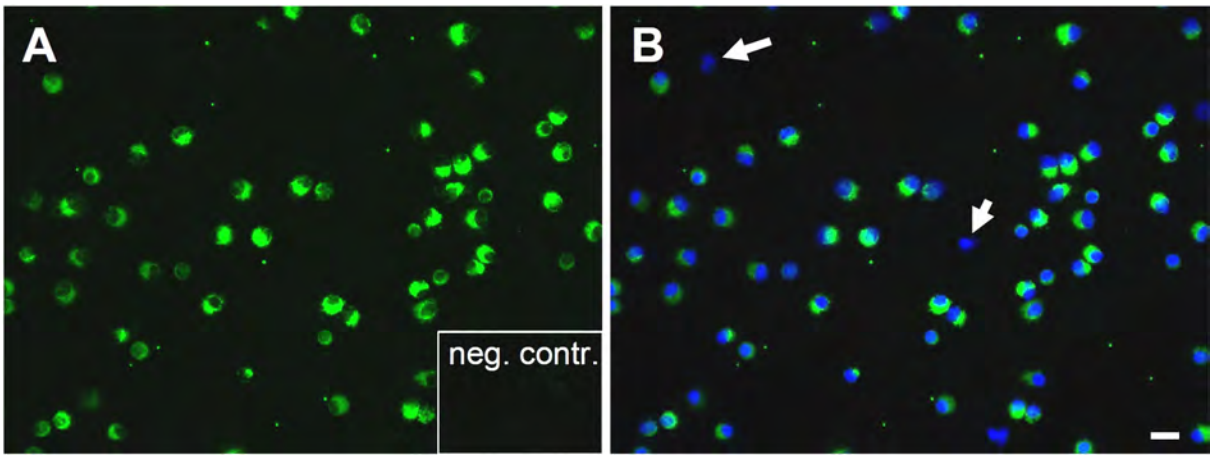

Fig. 3.1 Purity of isolated rat AM.
(A) Cells were immunolabeled with ED1 antibodies, an AM marker. (B) Merged image where nuclei of all cells present in the preparation were stained with DAPI. The majority of cells show a strong ED1 staining. Rare ED1-negative cells are indicated by arrows. Negative controls (neg. contr.) were done by omission of the primary antibody. Bar: 20 µm

3.1.2 Expression of nAChR analyzed by RT-PCR

RT-PCR analysis of mRNA isolated from BAL cells revealed expression of nAChR subunits α2, α3, α5, α9, α10, β1, and β2. Products corresponding to the α4, α6, α7, and β3 subunits were never found in BAL cells (Fig. 3.2), although they were easily detectable in DRG and lung homogenate. The mRNAs of subunits α9, α10, β1, and β2 were consistently expressed. Subunits α2, α3, and β4 were detected in 1 out of 9 preparations. Subunit α5 was present in 3 out of 9 preparations. The identity of amplified products was confirmed by sequencing, and control runs without template were negative.

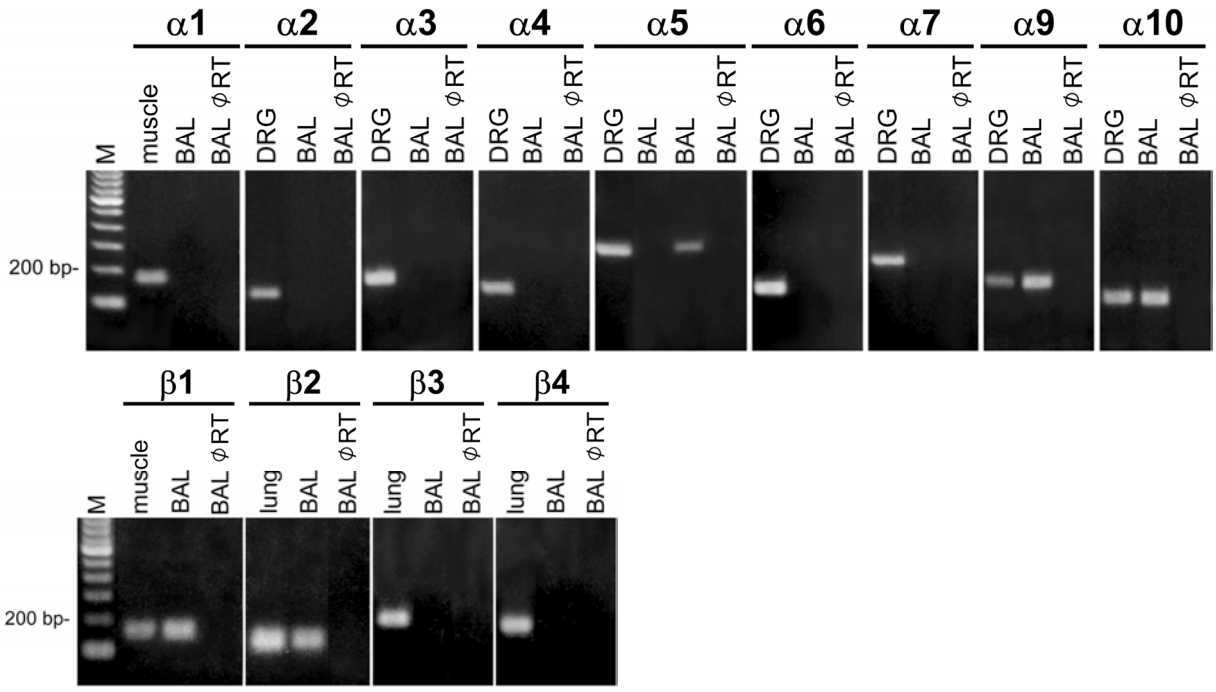

Fig. 3.2 RT-PCR analysis of nAChR subunits in rat BAL cells.

BAL cells consistently express mRNA coding for α9, α10, β1 and β2 subunits. Subunit α5 has an interindividual expression pattern, as shown in the two different BAL samples. The mRNAs coding for α1, α4, α6, α7 and β3 subunits are absent. Positive controls were run on DRG (α2-α10), lung (β2-4), and skeletal muscle (α1 and β1). Negative controls were done without RT. M = DNA marker

3.1.3 Immunocytochemistry for nAChR

Expression of nAChR receptors was assessed by immunocytochemistry. ED1-positive cells were immunoreactive for α9 and α10 nAChR subunits, and in 50% of BAL preparations (5 out of 10 samples) for the α5 subunit as well (Fig. 3.3). Preabsorption with the synthetic peptides used for immunization resulted in absence of immunolabeling for α5 and α9 subunits. Specificity of α10 nAChR subunit antibody was tested by western blotting (see 3.1.4 and Fig. 3.7). Staining was predominantly intracellular, localized near the nucleus, except for α5 and α9 subunit-immunoreactivity that exhibited a punctate surface distribution in a subset of AM (Fig. 3.3, inserts). The rabbit polyclonal antibody to α7 subunit faintly stained AM, but preabsorption with the corresponding peptide gave the same staining pattern. The monoclonal antibody to the α7 nAChR subunit (mAb 306) did not show any labeling of AM. Positive controls were run on DRG sections, demonstrating labeling of neuronal cell bodies with mAb

306 (Fig. 3.4). Similar staining patterns with antisera directed against nAChR subunits were observed in rat lung sections (Fig. 3.4). Here, the antiserum against the α9 nAChR subunit showed marked punctuate membrane staining of AM (Fig. 3.4, *insert*). There was no specific labeling for subunits α3 and α4 in ED1-positive cells.

Immunofluorescence with antisera directed against β2 and β3 nAChR subunits showed strong staining of ED1-positive BAL cells, while antibodies to the β4 subunit gave faint staining (Fig. 3.5A). Similar staining patterns were noted in rat lung sections, but preabsorption controls with peptides corresponding to β3 and β4 nAChR subunits gave the same signal intensity in ED1-positive cells (Fig 3.5B). Preabsorption control for β2 nAChR antibodies showed slighty less intense labeling of AM (Fig. 3.5B).

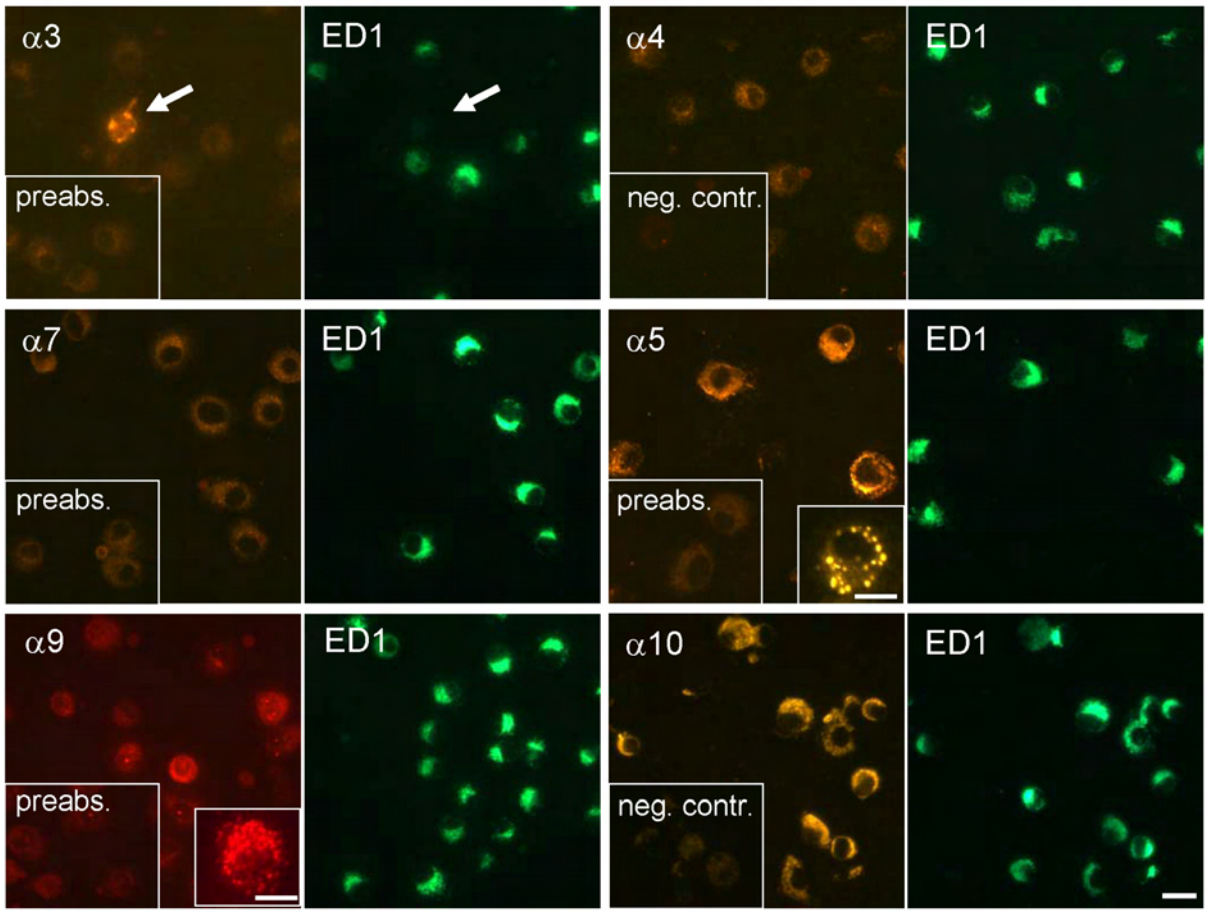

Fig. 3.3 Double-labeling immunofluorescence for nAChR α-subunits

demonstrating the presence of the nAChR subunits α5, α9 and α10 in ED1-positive BAL cells. A punctate fluorescence pattern for the α5 and α9 nAChR subunits was found in a subset of AM (inserts). Negative results were noted for α3, α4 and α7 subunits in ED1-positive cells, despite occasional occurrence of α3 subunit immunoreactive ED1-negative cells (arrow). Negative controls (neg. contr.) were done by omission of the primary antibody. Preabsorption (preabs.) was done by incubation of the primary antibody with the peptide used to raise the antibody. Bar: 20 µm, 10 µm in inserts.

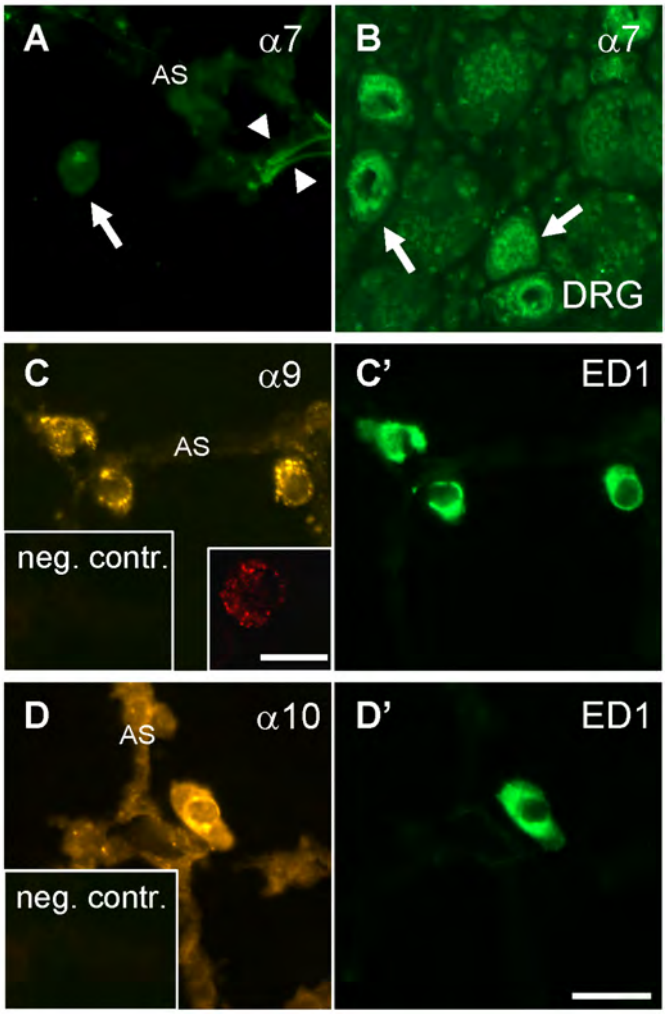

Fig. 3.4 Immunohistochemistry for nAChR α-subunits on rat lung and DRG sections. The mAb 306 directed against the α7 subunit fails to label AM *(A, arrow)* but it stains nerve cell bodies *(B, arrows)* in DRG sections serving as a positive control. *Arrowheads:* Elastin autofluorescence in alveolar septum. The antibodies to α9 (C) and α10 (D) subunits label ED1-positive AM in alveoli. The *insert* demonstrates punctate α9 subunit immunoreactivity in a CLSM optical section. Negative controls (neg. contr.) were done by omission of the primary antibody. *AS* = alveolar septum. Bar: 20 μm

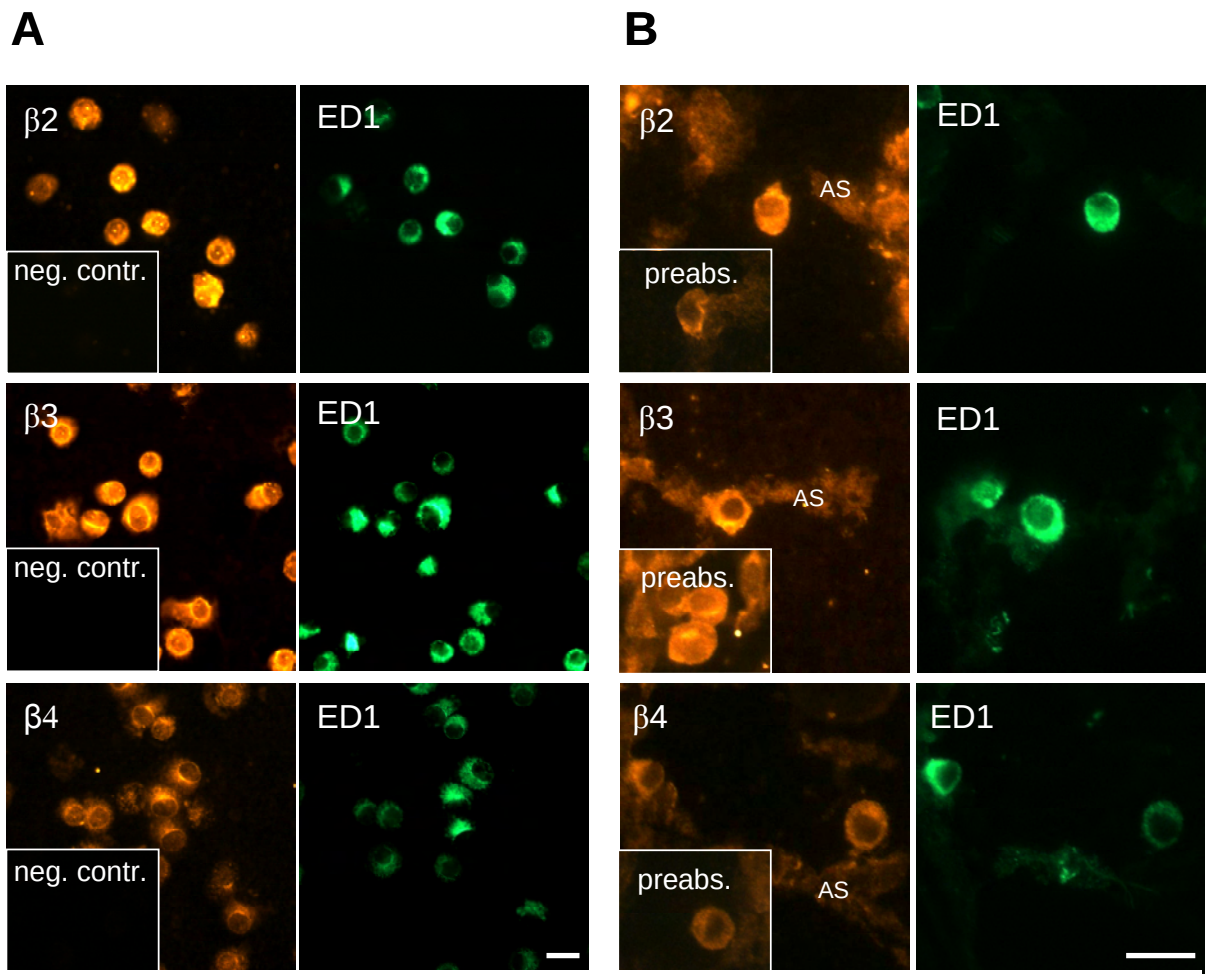

Fig. 3.5 Double-labeling immunofluorescence with antibodies for nAChR β-subunits. Antisera directed against β2, β3 and β4 reacted with ED1-positive cells in BAL samples (A) and rat lung sections (B). However, labeling could not be fully preabsorbed with peptides used to generate the antibodies. Negative controls (neg. contr.) were done by omission of the primary antibody. Preabsorption (preabs.) was done by incubation of the primary antibody with the peptide used to raise the antibody. Bar: 20 μm

3.1.4 Western blotting

Western blotting supported the immunohistochemical findings in that the mAb 306 failed to detect the α7 subunit in protein preparations from BAL cells while it recognized a ~50 kDa band in protein extracts from rat brain (Fig. 3.6). In Western blots of rat skin homogenates serving as a positive control, a single 67-kDa protein was recognized utilizing a previously characterized antiserum directed against the α10 nAChR subunit [95]. As shown in Fig. 3.6, this band plus additional bands at 110-130 kDa were immunolabeled in protein preparations from BAL cells.

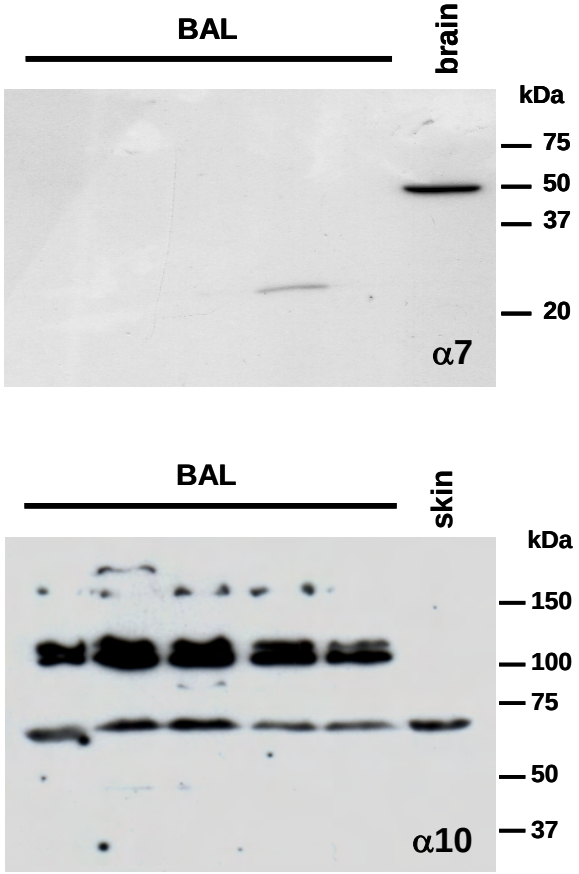

Fig. 3.6 Immunoblots.

No α7 subunit-immunolabeling is present in BAL samples, while the antibody mAb 306 recognizes a single 50 kDa protein band in protein extracts from rat brain. Affinity purified polyclonal antibodies to α10 nAChR label a protein band at 67 kDa in BAL cells and rat skin samples. In BAL cells, additional bands at 110-120 kDa are immunolabeled.

3.2 *Functional analysis of nAChR in rat alveolar macrophages*

3.2.1 ATP-triggered increase in Ca^{2+} derives from intracellular stores

In freshly isolated rat AM, ATP (10^{-4} M) induced a rise in $[Ca^{2+}]_i$ which reached maximum within 10 s after drug addition, and was followed by a slow decrease. Exclusion of calcium ions from the external solution had no effect on the maximal amplitude of the ATP-induced $[Ca^{2+}]_i$ rise (46% for $+Ca^{2+}$ and 47% for $–Ca^{2+}$ Hepes buffer). Macrophages exposed to extracellular Ca^{2+} showed a sustained increase in $[Ca^{2+}]_i$ whereas cells stimulated in Ca^{2+}-free buffer showed only a transient rise without reaching a phase of elevated $[Ca^{2+}]_i$ ($P \leq 0.001$) (Fig. 3.7A). The percentage of cells reacting to the ATP stimulus was decreased from 58% in Ca^{2+}-containing to 18% in Ca^{2+}-free buffer (582/1012 cells in $+Ca^{2+}$ vs 197/1085 cells in $–Ca^{2+}$ buffer). To better understand which receptors might be involved in ATP-induced $[Ca^{2+}]_i$ rise, we performed RT-PCR analysis and found $P2Y_1$, $P2Y_2$ and $P2Y_4$ purinergic receptor mRNAs in AM (Fig. 3.7B).

3.2.2 Nicotine modulates ATP-induced rise in $[Ca^{2+}]_i$

We tested whether nicotine modulates $[Ca^{2+}]_i$ levels. Half of the macrophage population (131/261 cells) exposed to ATP showed a transient rise in $[Ca^{2+}]_i$ (increase by 47%). Application of nicotine (10^{-6}, 10^{-5}, 10^{-4} M) or epibatidine (10^{-6} M) had no direct effect on $[Ca^{2+}]_i$. Nicotine given 2 min prior to ATP reduced the ATP-induced calcium peak by 38% ($P \leq 0.006$), while not changing the percentage of cells reacting to the ATP stimulus (54%, 120/222 cells). Epibatidine was neither effective in reducing ATP-induced transients nor it changed the percentage of cells reacting to the ATP (51%, 117/230 cells) (Fig. 3.8A).

In a separate set of experiments, we tested if the effect of nicotine can be blocked with the α1, α7 and α9/α9α10 nAChR antagonist α-bungarotoxin. This drug alone (10^{-7} M) had no effect on ATP-induced calcium increase, when compared to vehicle control, but it abrogated the effects of nicotine ($P \leq 0.001$). The transient rise in $[Ca^{2+}]_i$ in cells treated with α-bungarotoxin together with nicotine was slightly increased compared to vehicle-treated cells ($P \leq 0.025$) (Fig. 3.8).

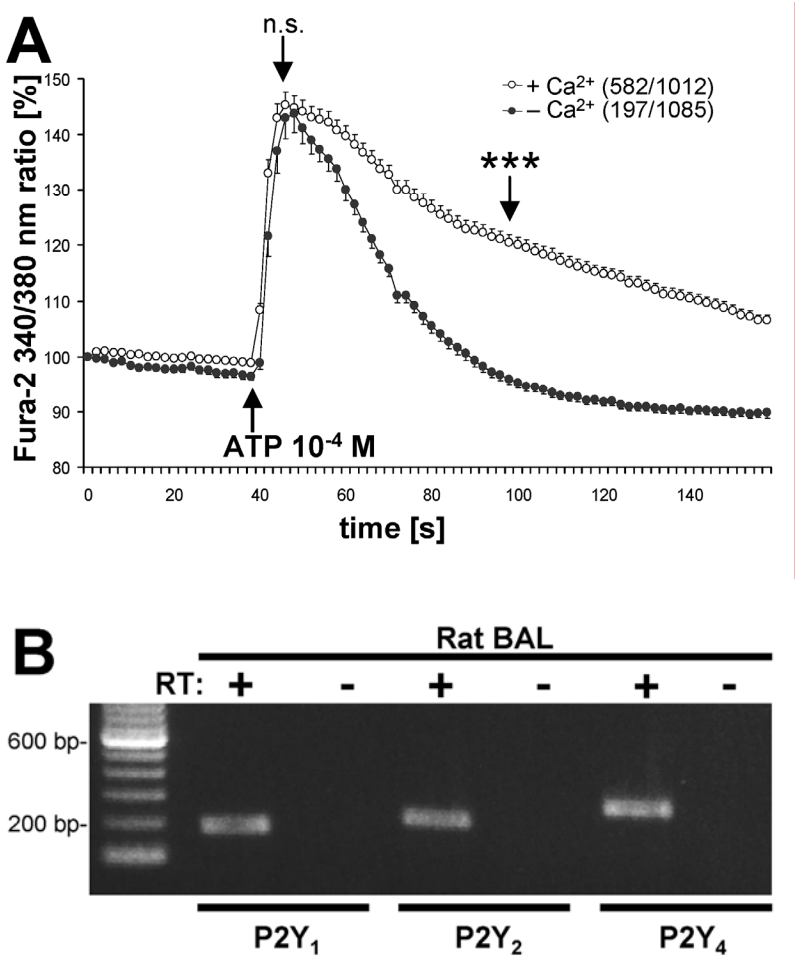

Fig. 3.7 Purinergic receptors on freshly isolated BAL cells.

ATP-induced transients in $[Ca^{2+}]_i$ are mainly dependent on intracellular stores. (A) Ratiometric $[Ca^{2+}]_i$ recordings. BAL cells were stimulated with ATP (10^{-4} M) in the presence or absence of extracellular Ca^{2+}. Sustained increase, but not initial rise in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+}. Number of cells which reacted, and total number of measured cells are given in brackets. Differences in signal intensity immediately before, and after 10 s or 1 min following the application of ATP were tested for significance. n.s. = not significant, ***$P \leq 0.001$. (B) RT-PCR in rat BAL cells shows expression of $P2Y_1$, $P2Y_2$ and $P2Y_4$ purinergic receptors.

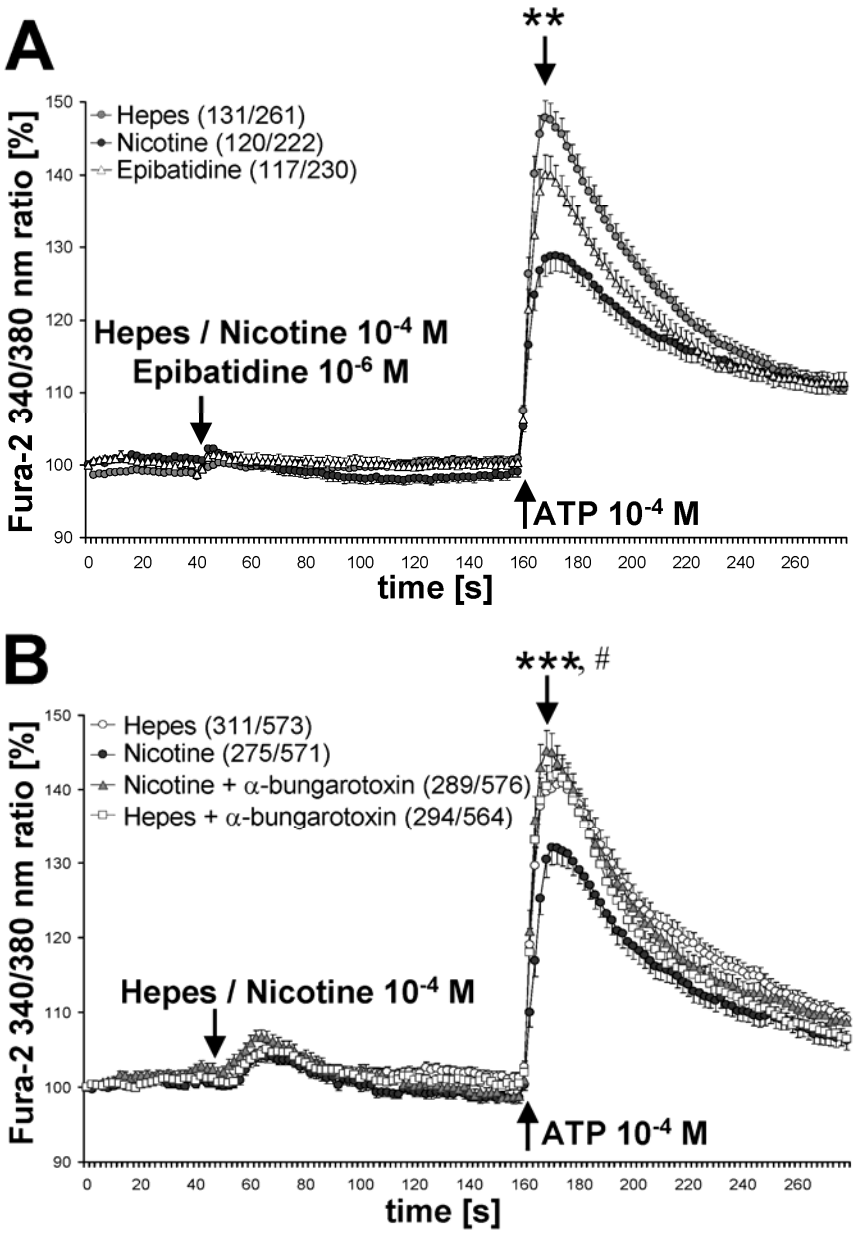

Fig. 3.8 Nicotine dampens ATP-induced increase in $[Ca^{2+}]_i$.

Ratiometric $[Ca^{2+}]_i$ recordings. (A) BAL cells were stimulated with ATP (10^{-4} M) in the presence or absence of nicotine (10^{-4} M) or epibatidine (10^{-6} M). ** $P \leq 0.01$ (nicotine compared to Hepes) (B) The reduction of ATP-induced rise in $[Ca^{2+}]_i$ by treatment with nicotine is blocked by pre-incubation with the nicotinic antagonist α-bungarotoxin (10^{-7} M). ***$P \leq 0.001$ (nicotine compared to α-bungarotoxin + nicotine), #$P \leq 0.05$ (Hepes compared to α-bungarotoxin + nicotine). Number of cells which reacted, and total number of measured cells are given in brackets. Differences in Fura-2 ratio immediately before and 10 s after the application of ATP were tested for significance.

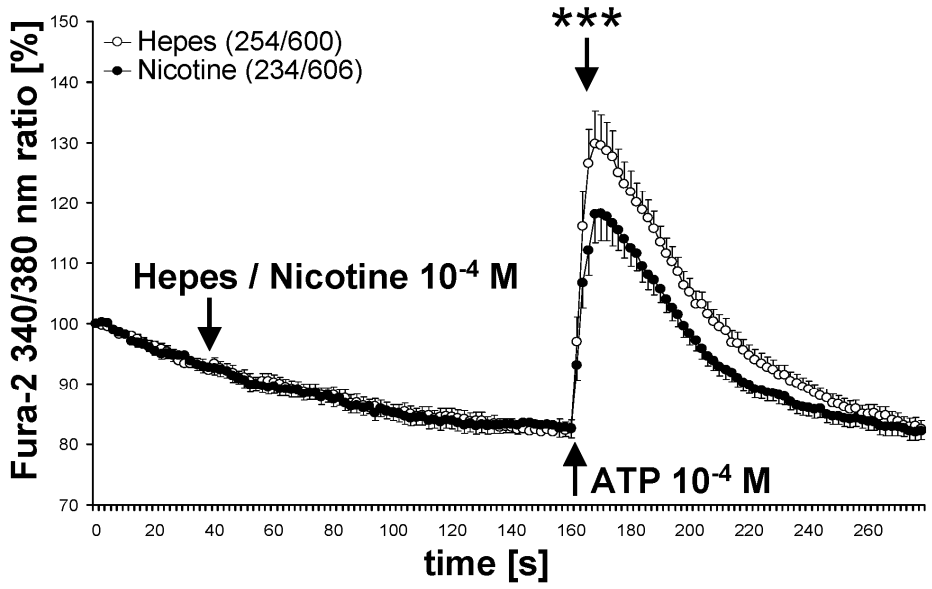

Fig. 3.9 Nicotine-mediated effect on ATP-induced $[Ca^{2+}]_i$ rise is not depended on extracellular calcium.

Ratiometric $[Ca^{2+}]_i$ recordings. BAL cells were treated with nicotine (10^{-4} M) or with the vehicle in the presence of extracellular Ca^{2+}. BAL cells were pre-treated with nicotine or with the vehicle, and then stimulated with ATP (10^{-4} M). Number of cells which reacted, and total number of measured cells are given in brackets. Differences in Fura-2 ratio immediately before and 10 s after the application of ATP were tested for significance. ***$P \leq 0.001$.

3.2.3 Nicotinic modulation is not dependent on extracellular calcium

Next we tested if the nicotine-mediated effect upon the ATP-induced $[Ca^{2+}]_i$ rise is depended on extracellular calcium. Immediately before measurements, cells were transferred to Ca^{2+}-free buffer. In this condition, a constant decrease in $[Ca^{2+}]_i$ can be observed. Cells treated with nicotine (10^{-4} M) 2 min before the ATP stimulus showed a decreased amplitude of the ATP-induced rise in $[Ca^{2+}]_i$. This was not affected by the absence of Ca^{2+} in the external bath solution (Fig. 3.9). The number of cells reacting to ATP was reduced when Ca^{2+} was omitted in the external solution (18% for vehicle and 16% for nicotine treated cells) compared to Ca^{2+}-supplemented medium (42% for vehicle and 39% for nicotine treated cells).

3.3 *Expression of 5-HT receptors and nAChR on freshly isolated mouse alveolar macrophages*

3.3.1 Purity of isolated mouse alveolar macrophages

The majority of isolated BAL cells was identified as leukocytes by CD45 staining (Fig. 3.10B). These cells were CD11c+ and CD11b- (94.4%±3.35% of CD45+ cells). There was a small fraction of CD11c+/CD11b+ cells in BAL (Fig. 3.10C).

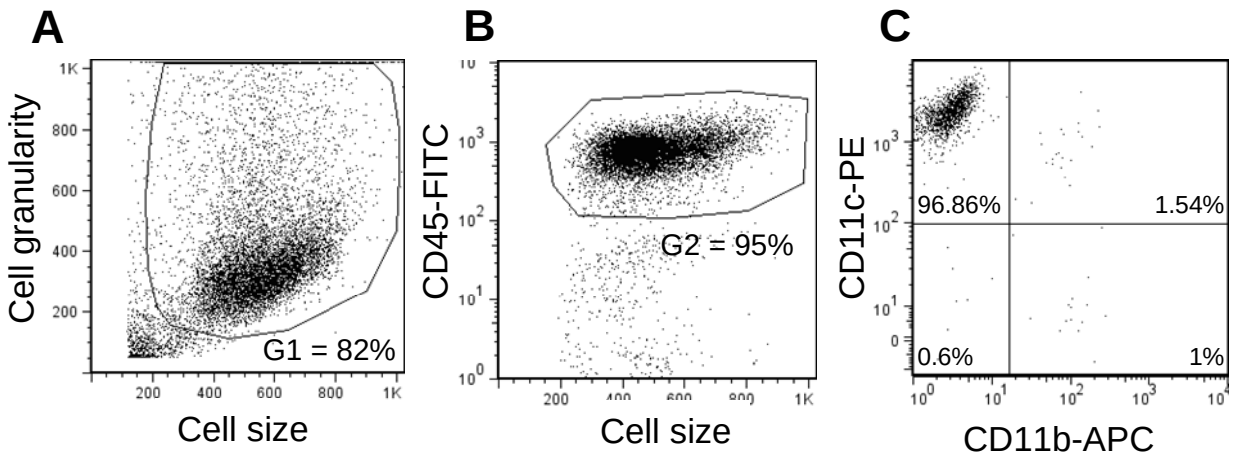

Fig. 3.10 Flow cytometry on BAL cells recovered from mouse lung.
Cells were gated as in (A) based on size and granularity (G1) and subjected to CD45-FITC staining (B). CD45+ cells were gated (G2) and further analyzed using CD11c and CD11b antibodies. Results are representative of n=4 experiments.

3.3.2 Expression of 5-HT receptors assessed by RT-PCR

All analyzed BAL samples expressed mRNA for the 5-HT_{2C} receptor. A faint bands corresponding to 5-HT_{2A} and 5-HT_{2B} receptor mRNAs were found in 3 out of 5 preparations. (Fig. 3.11).

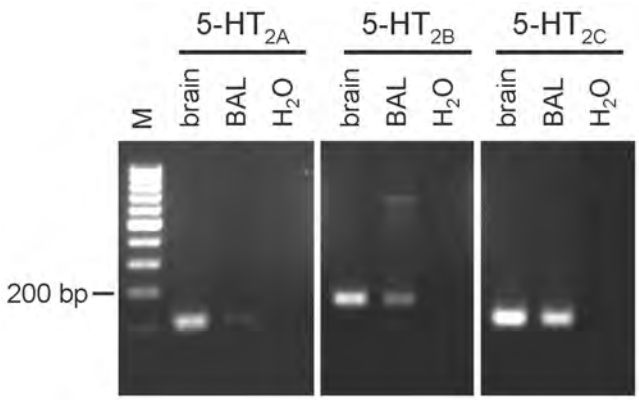

Fig. 3.11 Expression of 5-HT$_2$-receptors in mouse BAL cells.

All tested BAL preparations were positive for 5-HT$_{2C}$ receptor mRNA. The 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors were found to be expressed in 3 out of 5 samples. Brain was used as a positive control. M = DNA marker.

3.3.3 Expression of nAChR analyzed by RT-PCR

Freshly isolated mouse AM expressed mRNAs for nAChR subunits α9, β2 and β4 (Fig. 3.12). Messenger RNA for α10 subunit was found to be present in 2 out of 5 preparations. Products corresponding to the α7 subunits were never found in BAL cells (Fig. 3.12), although they were easily detectable in brain homogenate, serving as a positive control.

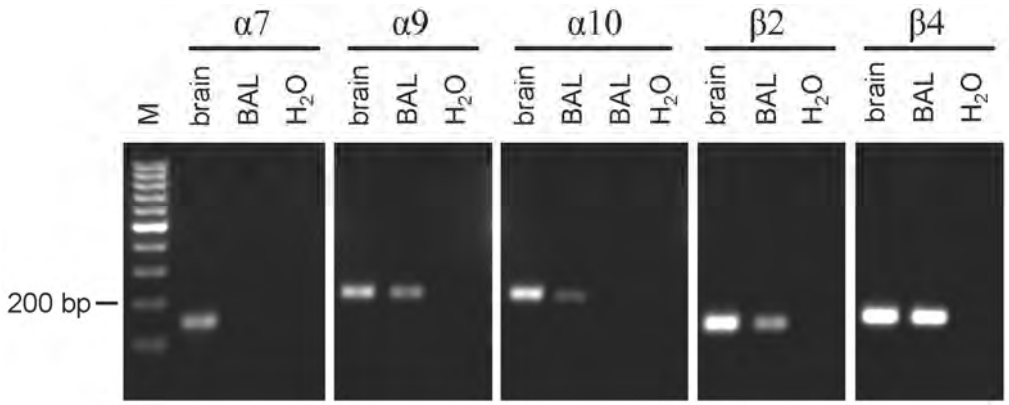

Fig. 3.12 RT-PCR analysis of nAChR subunits in mouse BAL cells.

BAL cells consistently express mRNAs coding for α9, β2 and β4 subunits. Expression of α10 subunit was seen in 2 out of 5 samples. The mRNA for α7 subunit was not found in AM preparations, although it is easily detectable in brain homogenate, serving as a positive control. M = DNA marker

3.3.4 Suitability of the 5-HT_{2C} receptor antibody for immunohistochemistry

Previous reports suggested sc-15081 antibody as being specific for 5-HT_{2C} receptors [97]. The suitability of this antibody was tested by immunolabeling of cryostat sections of 5-HT_{2C} receptor deficient and wild-type lungs. The antiserum produced identical immunohistochemical labeling patterns in tissues taken from wild-type and 5-HT_{2C} receptor-deficient mice (Fig. 3.13).

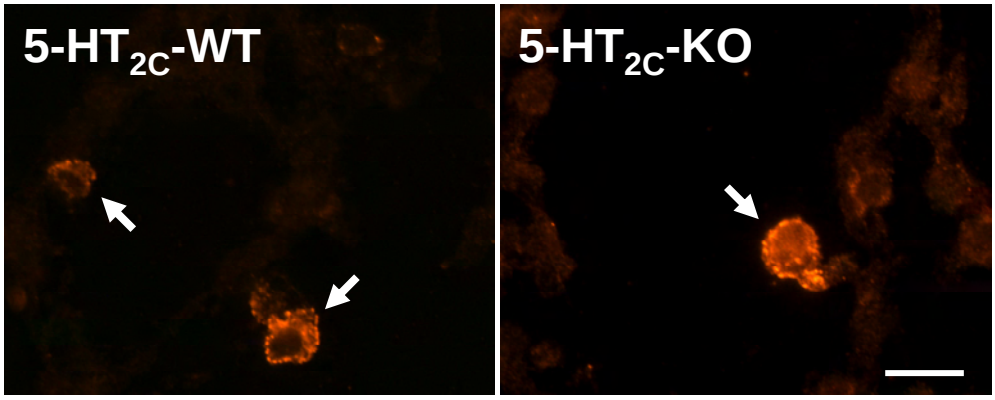

Fig. 3.13 Immunohistochemistry with sc-15081 antibody on mouse lung cryostat sections.

Identical pattern of labeling was observed in AM (*arrows*) from wild-type (5-HT_{2C}-WT) and 5-HT_{2C} receptor-deficient (5-HT_{2C}-KO) mice. Bar: 20 μm.

3.4 *Function of 5-HT receptors and nAChR on freshly isolated mouse alveolar macrophages*

3.4.1 5-HT potently stimulates $[Ca^{2+}]_i$ rise

In freshly isolated mouse AM, 5-HT (10^{-5} M) induced an immediate rise in $[Ca^{2+}]_i$ reaching its maximum in 6 s, followed by a brief, 15 s decrease and subsequent phase of increased $[Ca^{2+}]_i$ level (> 5 min). This effect was compared to that of a stimulation with ATP (10^{-4} M), an agonist of P2Y receptors expressed on AM [23]. Treatment with ATP resulted in a lower and shorter rise in $[Ca^{2+}]_i$ (Fig. 3.15A).

To examine the requirement of extracellular Ca^{2+} for rise in $[Ca^{2+}]_i$, AM were exposed to 5-HT in Ca^{2+}-free buffer. Alveolar macrophages exhibited only a transient rise without reaching a plateau phase (Fig. 3.15B). The peak increase in response to 5-HT was reduced by 35% in Ca^{2+}-free buffer when compared to Ca^{2+}-containing buffer. Accordingly, the ratio of Fura-2

340/380 nm fluorescence measured at the beginning of the recordings was lower by 23% in cells bathed in Ca^{2+}-free buffer (179±3 in Ca^{2+}-containing vs. 139±2 in Ca^{2+}-free buffer, $P \leq 0.001$).

We evaluated if 5-HT induces an increase in $[Ca^{2+}]_i$ in rat NR8383 AM cells. Responses to 5-HT (10^{-8}-10^{-5} M) were tested in n=3 cell preparations. We used a range of experimental designs (different agonist concentration, agonist given alone or in series of increasing concentration, preparation of cells which were cultured in medium containing 10% FCS or 10% dialysed FCS). It was unfeasible to group the data from all experiments; therefore a representative result is provided. Cells responded very weakly to 5-HT (10^{-5} M), while ATP induced a rapid elevation of $[Ca^{2+}]_i$ (Fig. 3.15).

3.4.2 Nicotine dampens 5-HT- and ATP-induced $[Ca^{2+}]_i$ rise

We tested if nicotine modulates $[Ca^{2+}]_i$ in mouse AM. Application of nicotine (10^{-4} M) had no direct effect on $[Ca^{2+}]_i$. Nicotine given 2 min prior to 5-HT and ATP, reduced the agonist-induced calcium peak by 19% and 9%, respectively ($P \leq 0.001$) (Fig. 3.16).

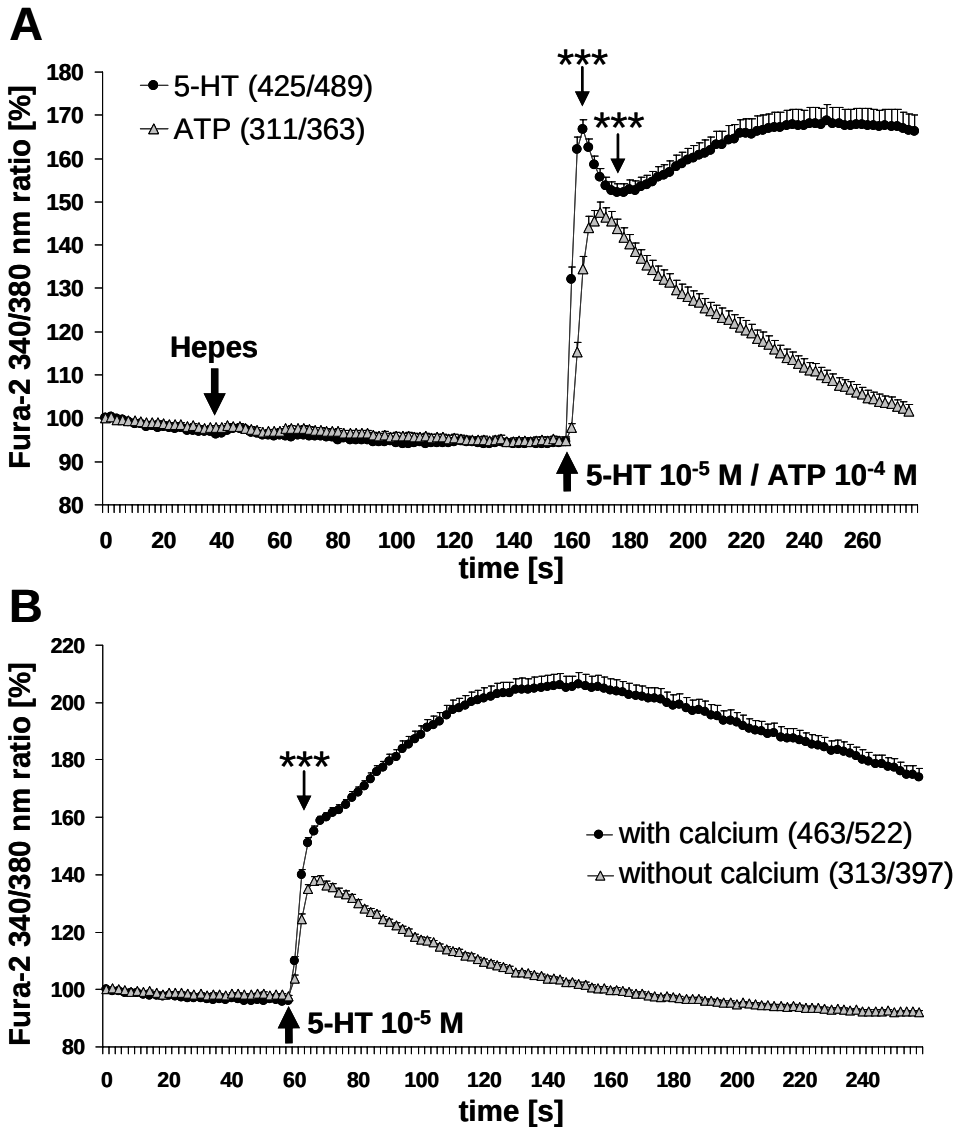

Fig. 3.14 5-HT induces Ca^{2+} release from intracellular stores followed by extracellular Ca^{2+} influx.

Ratiometric $[Ca^{2+}]_i$ recordings. A) 5-HT (10^{-5} M) induces a fast rise in $[Ca^{2+}]_i$ with a subsequent short decline and then a phase of elevated $[Ca^{2+}]_i$ levels. In AM stimulated with ATP (10^{-4} M), the Ca^{2+} response is weaker when compared to 5-HT. (B) Dependency of the 5-HT-induced calcium increase on extracellular calcium. Cells exposed to 5-HT (10^{-5} M) in Ca^{2+}-containing buffer show sustained increase in $[Ca^{2+}]_i$ whereas cells stimulated in buffer without Ca^{2+} and supplemented with EGTA (3×10^{-6}) exhibit only a transient rise in $[Ca^{2+}]_i$. Number of cells which reacted, and total number of measured cells are given in brackets. Differences in Fura-2 ratio immediately before application of agonist and after 6 or 20 s were tested for significance. *** indicates $P \leq 0.001$.

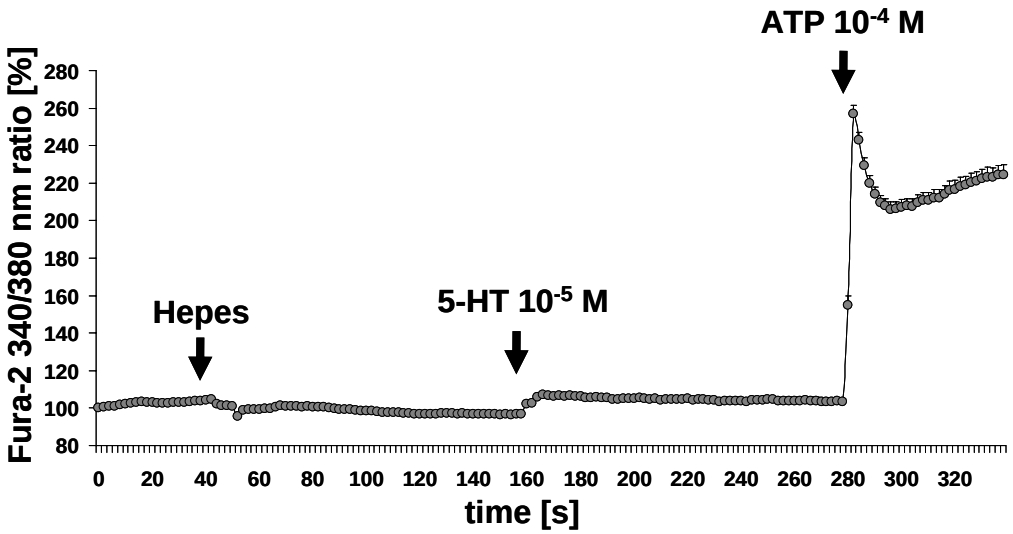

Fig. 3.15 5-HT has only a small effect on $[Ca^{2+}]_i$ in NR8383 rat AM cells.

Ratiometric $[Ca^{2+}]_i$ recordings. NR8383 cells were stimulated with 5-HT (10^{-5} M) and ATP (10^{-4} M). Hepes buffer was used as a vehicle control. Depicted are recordings of $[Ca^{2+}]_i$ in 191 cells.

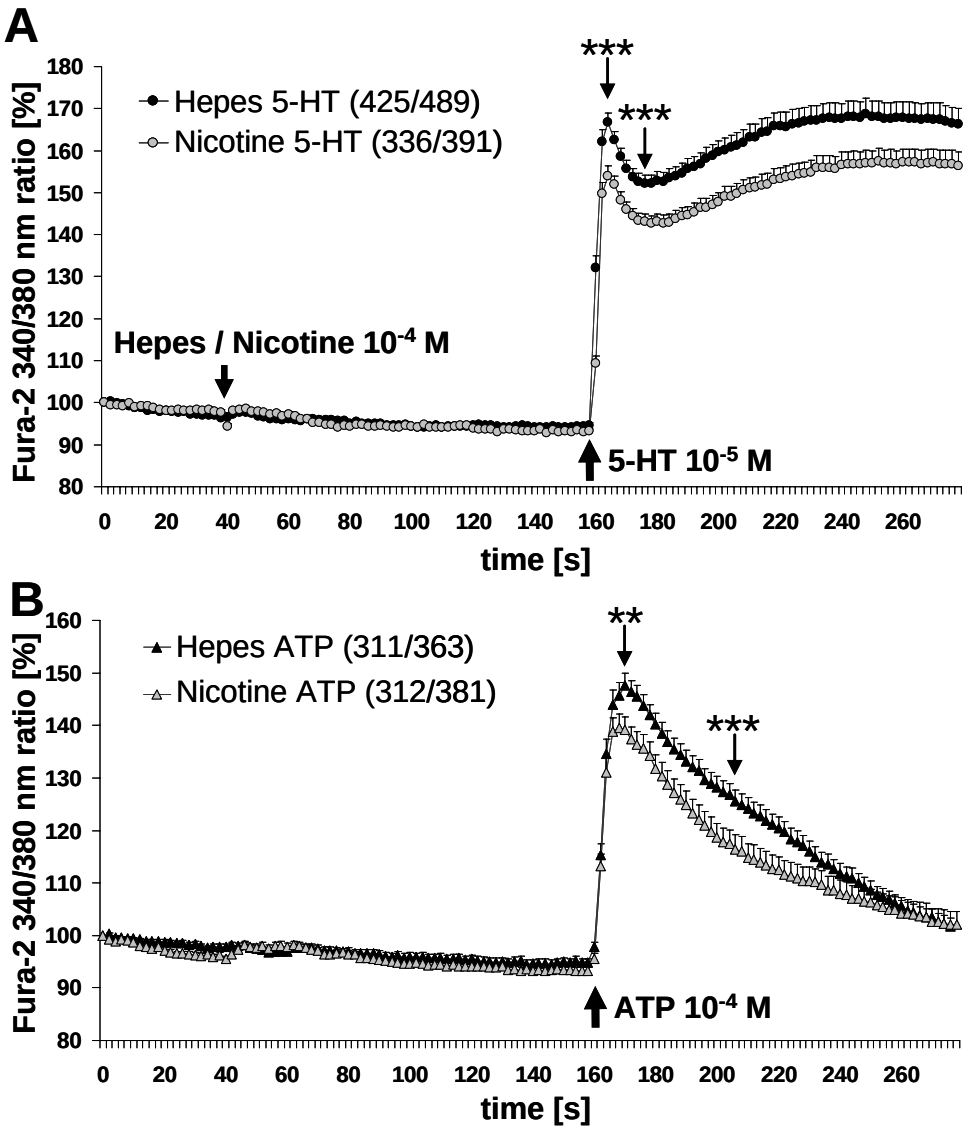

Fig. 3.16 Nicotine dampens agonist-induced increase in $[Ca^{2+}]_i$.

Ratiometric $[Ca^{2+}]_i$ recordings. Mouse BAL cells were stimulated with 5-HT (10^{-5} M) (A) or ATP (10^{-4} M) (B) in the presence or absence of nicotine (10^{-4} M). Number of cells which reacted, and total number of measured cells are given in brackets. Differences in Fura-2 ratio immediately before application of agonist and after time indicated by arrows were tested for significance. *** indicates $P \leq 0.001$. ** $P \leq 0.01$.

3.4.3 The 5-HT_{2C} receptor is required for 5-HT-induced rise in $[Ca^{2+}]_i$

To test whether the 5-HT-induced increase in $[Ca^{2+}]_i$ is mediated by the 5-HT_{2C} receptor, AM were incubated with the selective 5-HT_{2C} blocker RS102221 (10^{-9} M) 2 min prior to stimulation with 5-HT. This markedly inhibited the 5-HT rise in $[Ca^{2+}]_i$ without interfering with the ATP effect (Fig. 3.17A).

Accordingly, the 5-HT effect was absent in 5-HT_{2C}-receptor deficient animals. In the wild-type AM, 5-HT induced a rapid rise in $[Ca^{2+}]_i$, whereas it had no effect on this parameter in the 5-HT_{2C} receptor-deficient cells. Alveolar macrophages from both mouse strains were normally reactive to ATP, which served as a positive control (Fig. 3.17B).

3.4.4 5-HT modulates chemokine and cytokine production

Cytokine and chemokine production in AM was screened with a dot-blot based assay ("Mouse Cytokine Array"). In AM stimulated with 5-HT (10^{-5} M) for 24 h, there was an increase in signal intensity over unstimulated control for CCL2, and to a lesser degree for CCL5, TNFα, and CXCL1 proteins (Fig. 3.18).

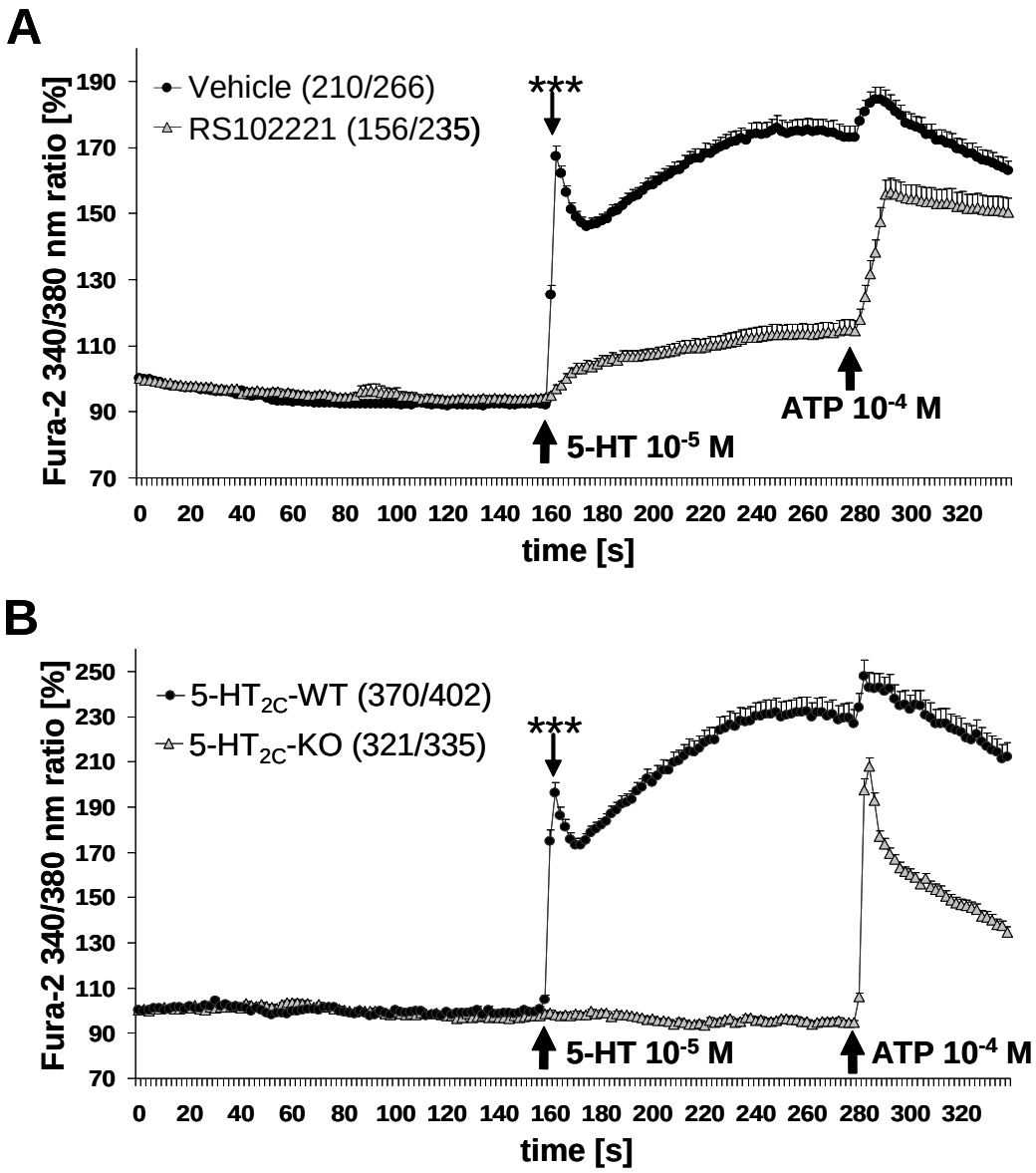

Fig. 3.17 5-HT$_{2C}$ receptors are required for 5-HT-induced [Ca^{2+}]$_i$ rise.

Recordings of [Ca^{2+}]$_i$ in AM. (A) Cells were incubated with the 5-HT$_{2C}$ receptor antagonist RS-102221 (10^{-9} M) 2 min prior to stimulation with 5-HT (10^{-5} M). Response to the 5-HT stimulus is blocked in treated cells, but the reaction to ATP is not blunted by the drug. (B) Cells harvested from 5-HT$_{2C}$ receptor deficient animals (5-HT$_{2C}$-KO) and from littermate controls (5-HT$_{2C}$-WT) were stimulated with 5-HT (10^{-5} M) and ATP (10^{-4} M). In wild-type cells, 5-HT induces [Ca^{2+}]$_i$ rise which was absent in KO cells. All cells were reactive to ATP, serving as a positive control. Number of cells which reacted, and total number of measured cells are given in brackets. Differences in Fura-2 ratio immediately before application of agonist and after time indicated by arrows were tested for significance. *** indicates $p<0.001$.

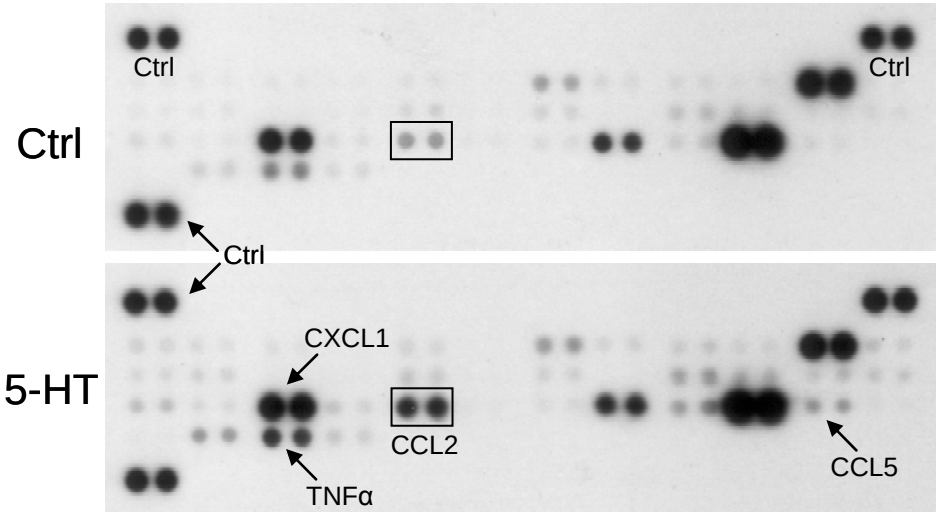

Fig. 3.18 CCL2, CCL5, TNF*α*, and CXCL1 expression is increased in AM upon 5-HT stimulation.

Mouse cytokine dot blot array was used to detect changes in AM secretions which were treated with 10^{-5} M 5-HT for 24 h (n=2). Ctrl = control spots.

4 Discussion

Alveolar macrophages hold a key position in initiating pulmonary inflammatory responses by secreting TNFα and several additional cytokines and chemokines. These events are tightly regulated at the level of transcription, translation and secretion. Immune cells sense and signal to their environment by means of soluble mediators secreted by activated cells. In recent years, mediators that are primarily associated with neurotransmission have been recognized as potent modulators of immune cells. This thesis was focused on investigating acute cellular effects of nicotine and 5-HT upon AM.

4.1 *Modulation of alveolar macrophages by nAChR signaling*

We demonstrated acute receptor-dependent, modulatory effects of nicotine on AM. The nAChR involved in this process differ from subtypes reported previously to be involved in "cholinergic anti-inflammatory pathways" outside the lung. Although the effects of nicotine are receptor mediated, these receptors do not form a classical ion channel known from neuronal cells. Importantly, we were not able to detect the mRNA coding for α7 nAChR in both mouse and rat AM although this subunit was easily detectable in sensory neurons and in whole lung and brain homogenate. Immunofluorescence with two commercially available antibodies to nAChR α7 and immunoblotting with the monoclonal antibody mAb 306 on rat AM supported this finding. This is consistent with reported data on the lack of the expression of α7 nAChR in the murine AM cell line MH-S [61] and our previous work on expression of nAChR in freshly isolated murine AM [62] from FVB mice. In contrast, α7 nAChR are essential for systemic cholinergic anti-inflammation since the beneficial effects of nicotine in endotoxemia are abrogated in α7 subunit gene-deficient mice [64]. Accordingly, two potent α7 nAChR agonists, GTS-21 and PNU-282987 [98, 99], inhibit LPS-induced TNFα release and reduce acid-induced acute lung injury, respectively, in the mouse lung [68, 100]. Their potency on the most prevalent nAChR subunits identified in our present study on AM, i.e. α9 and α10 nAChR that generally share many pharmacological properties with α7 nAChR [101], yet has not been determined. Without doubt, however, α7 nAChR is expressed in the lung as demonstrated by RT-PCR in this and previous studies [102, 103]. Functional data show increases in acid-induced excess lung water and vascular permeability in α7 nAChR deficient mice [68]. Nicotinic receptors located on endothelial cells may account for this effect [104]. However, since all α7 nAChR antibodies tested so far produce immunohistochemical labeling also in organs taken from α7 nAChR deficient mice [60, 105], immunohistochemistry alone

cannot decipher the cell-type specific α7-subunit distribution in the lung, and this issue remains to be solved.

Instead of α7 we observed expression of nAChR subunits α9, α10, β1, and β2, and to a variable extent α2, α3, α5, in rat AM. Mouse AM expressed α9, α10, β2, and β4 nAChR subunits. In rat AM samples, we were able to amplify mRNA coding for α9 subunit only with SuperScriptII and not with iScript as reverse transcriptase system. This may be due to different priming strategies (oligo(dT) and blend of oligo(dT) + random hexamer primers, respectively) or to reduced RNAse H activity in SuperScriptII enzyme, which enables more efficient cDNA synthesis [106]. To form classical, ion-conducting nAChR, α subunits combine as heteropentamers with β subunits or build α heteropentamers of α9α10 and homopentamers of α7 and α9 (for a review, see [107]). The subunits detected in rat AM in the present study would allow combining the following nAChR pentamers: α3β2, α3α5β2, α9α10, and α9 as homopentamer. Since there is a constant expression of subunits α9 and α10 in AM, this combination as homo- or heteropentamer seems to be most likely, if pentamer formation occurs at all.

These subunits have been best characterized in the inner ear, where they form Ca^{2+}-permeable ion channels involved in efferent modulation of hair cell function [108, 109]. Interestingly, Ca^{2+} recordings in AM did not reveal changes in $[Ca^{2+}]_i$ in response to nicotine. This notion was supported by whole-cell patch clamp recordings of AM done by Gitte Jositsch from Prof. Dr. W. Clauss laboratory, which did not show any transmembrane currents in response to ACh. This data are included in our manuscript on the receptor-mediated nicotinic effect on AM [110]. Similarly, a subpopulation of human T-lymphocytes expresses α9 and α10 nAChR subunits but fails to show transmembrane currents triggered by ACh [111], and nicotine does not cause alteration of $[Ca^{2+}]_i$ in the rat AM cell line NR8383 [112] and in rat intravascular mononuclear leukocytes obtained from isogenic kidney transplants [55]. Thus, α9α10 nAChR subunits apparently do not form classical ionotropic receptors in cells of the immune system. Still, α9α10 nAChR subunits confer intracellular effects as our data demonstrate an acute α-bungarotoxin sensitive modulatory effect of nicotine upon ATP-induced calcium release from intracellular stores. Similarly, we recently identified a methyllycaconitine sensitive modulatory effect of nicotine upon ATP-induced rise in $[Ca^{2+}]_i$ in rat mononuclear leukocytes obtained by vascular perfusion of isogenic kidney transplants [55]. In line with this observation, α9 subunit containing nAChR in outer hair cells of the inner ear do not exclusively assemble into ionotropic receptors, but form metabotropic receptors as well. Here,

ACh also reduces ATP-induced rise in $[Ca^{2+}]_i$ at a concentration that alone is insufficient to impact $[Ca^{2+}]_i$, and again this effect is α-bungarotoxin sensitive [113].

Atypical, non-ionotropic effects have also been reported for the nAChR α7 subunit. In T cells, this subunit fails to form a ligand-gated Ca^{2+} channel but interacts with CD3ζ to modulate TCR/CD3 function [54]. Notably, α7 subunits in this complex exhibit a different agonist/antagonists profile than neuronal ionotropic α7 nAChR. Methyllycaconitine and α-bungarotoxin, both potent inhibitors of ionotropic α7 nAChR, indeed are strong agonists at T cells expressing nAChR α7 subunits [54]. Correspondingly, epibatidine, a highly potent agonist at ionotropic nAChR, failed to mimic the nicotine effect in our present experiments on rat AM.

In contrast to the well-characterized channel properties of nAChR, the mechanisms of atypical nAChR signaling are currently only poorly understood. Membrane bound nAChR subunits have been demonstrated to interact with and to modulate signaling by β-arrestin [56], phosphatidyl-inositol-3-kinase [57], CD3ζ [54], and purinergic P2X-receptors [58, 59]. The latter are involved in ATP-induced increase in $[Ca^{2+}]_i$ by extracellular influx in human AM, since initial Ca^{2+} transients are reduced by 40% in Ca^{2+}-free medium [114]. In our present study of rat AM, however, the ATP-induced initial increase in $[Ca^{2+}]_i$ and the modulatory effect of nicotine persisted in Ca^{2+}-free solution, demonstrating interference of atypical nAChR with P2Y-receptor mediated Ca^{2+}-release from intracellular stores. In support, we observed expression of P2Y purinergic receptors on AM, among them P2Y2 that mediates Ca^{2+}-release from the ER in mouse macrophages [23]. Nicotinic receptors, whose mRNA was found in mouse AM, are also likely to mediate nicotine-induced decrease in response to ATP and 5-HT. Further studies are required to test if this nicotine effect is also receptor-dependent.

Extracellular ATP is well recognized as a “danger” or “host tissue damage” signal and is mostly regarded to promote inflammation [115, 116]. In human AM, it couples to $[Ca^{2+}]_i$ increases and stimulates IL-1β and IL-6 release albeit suppressing TNFα production [114]. In the rat AM cell line NR8383, ATP induces P2Y2- and Ca^{2+}-dependent increase in CCL2 synthesis and release [45]. As described in detail in chapter 4.2, the CCL2-CCR2 axis is a crucial regulator of inflammatory cell influx into the murine lung [5, 117]. Hence, the presently observed nicotinic attenuation of ATP-induced rise in $[Ca^{2+}]_i$ can be considered as an anti-inflammatory mechanism triggered by atypical nAChR.

In summary, AM are equipped with modulatory nAChR with properties distinct from ionotropic nAChR mediating synaptic transmission in the nervous system. Their stimulation with nicotine dampens ATP and 5-HT-induced Ca^{2+}-release from intracellular stores in both

rat and mouse AM. Thus, the present study identifies the first acute receptor-mediated but atypical nicotinic effect on AM with anti-inflammatory potential.

4.2 *Serotonin modulates alveolar macrophage function via the 5-HT$_{2C}$ receptor.*

The current thesis provides the first evidence for functional expression of 5-HT$_{2C}$ receptors on mouse AM. In freshly isolated AM, 5-HT rapidly stimulates a rise in $[Ca^{2+}]_i$ and leads to the increased production of CCL2, CCL5, TNFα.

While many studies have investigated effects of 5-HT on cytokine production in various immune cells, little is know about the action of 5-HT upon AM. Acute effects of 5-HT on AM have not been investigated so far. Previous studies pointed to the importance of 5-HT$_2$ receptors in the development of bleomycin-induced pulmonary fibrosis in mice [118] and monocrotaline-induced pulmonary hypertension in rats [119]. The first evidence of 5-HT$_{2C}$ receptor expression on AM derived from a microarray study [120].

In freshly isolated AM, RT-PCR revealed constitutive expression of 5-HT$_{2C}$ receptor mRNA while the two other 5-HT$_2$ receptors were found in lower amounts. In contrast to previous report by Bubbar *et al.* [97], the polyclonal antibody sc-15081 directed against the 5-HT$_{2C}$ receptor failed to produce specific labeling, as evidenced from experiments with wild-type and 5-HT$_{2C}$ receptor-deficient mouse lung tissue sections. This finding may be explained by use of different batches of polyclonal antibody, which may differ in specificity.

The presence and function of 5-HT$_{2C}$ receptors in AM were verified by $[Ca^{2+}]_i$ measurements utilizing the Fura-2 assay. Stimulation with 5-HT resulted in a rapid increase in $[Ca^{2+}]_i$, characterized by a fast initial rise in $[Ca^{2+}]_i$ and a subsequent phase of elevated Ca^{2+} levels, which was dependent on extracellular Ca^{2+}. The initial 5-HT-induced increase was reduced in AM bathed in buffer lacking Ca^{2+}. This might be caused by release of Ca^{2+} into the buffer, which may cause partial depletion of the intracellular Ca^{2+} stores. Indeed, mouse AM which were transferred to Ca^{2+}-free buffer show a reduction in 340/380 nm Fura-2 ratio values when compared to cells kept in Hepes buffer with Ca^{2+}. It is interesting that rat AM seem to be more resistant to decreased extracellular Ca^{2+} concentration, since in these cells we could not detect any differences in ATP-mediated $[Ca^{2+}]_i$ rise in buffers with or without Ca^{2+}. Therefore, it is likely that the initial rise in response to 5-HT relies on Ca^{2+} being mobilized from intracellular stores. The nature of the subsequent transmembrane influx of Ca^{2+} was not fully investigated, but it is likely that it occurs through mechanisms not related to ionotropic 5-HT receptors. Experiments with the selective 5-HT$_{2C}$ antagonist, RS102221 [121], showed that

this receptor is most likely responsible for the 5-HT-induced $[Ca^{2+}]_i$ increase. To confirm this finding, as well as to prove the existence of 5-HT_{2C} receptor on protein level, we used AM isolated from 5-HT_{2C} receptor-deficient animals. These cells do not respond to 5-HT but react to other G-protein coupled receptor agonist, i.e. ATP. Thus, it is very likely that the deficiency in Ca^{2+} response to 5-HT is solely due to lack of its receptor and not to defects in signal transduction downstream of the receptor.

As it has been shown in the section 1.5, changes in $[Ca^{2+}]_i$ in macrophages have been associated with multiple functions. To investigate physiological responses of AM upon 5-HT stimulation we measured secretions of AM by a dot-blot based assay. Indeed, changes in production of inflammatory mediators were observed. Mainly CCL2, but also CCL5, TNFα, and CXCL1 production were increased by 5-HT stimulation. A limitation of the assay is its low dynamic range, therefore increase in expression of CCL2 upon 5-HT treatment was confirmed with ELISA (data shown in [122]).

TNFα can be considered as the prototype of a large family of structurally related cytokines, which play a central role in the regulation of immune response [123]. The in vivo neutralization experiments with anti-TNFα antibodies demonstrated that the host defense against pathogens is severely impaired in the absence of TNFα [123, 124]. TNFα activates macrophages and neutrophils, stimulates the respiratory burst, and induces the production of leukocyte and vascular adhesion molecules, leading to enhanced transmigration into inflammatory sites [124]. In a murine model of *Klebsiella* spp pneumonia, in vivo neutralization of TNFα resulted in a 50% decrease in neutrophil influx to the lung with concomitant increase in number of bacteria recovered from lung and plasma, leading to increase in mortality (from 20% to 80%) [124]. However, excessive TNFα production is deleterious to the host. The presented data show that stimulation with 5-HT (10^{-5} M) increased production of TNFα in freshly isolated AM. Interestingly, in the rat NR8383 AM cell line as well as in human AM, 5-HT (10^{-10} M) decreased spontaneous as well as LPS-induced TNFα release [91]. This may suggest that 5-HT differentially modulates cellular responses in broad range of concentrations by acting on different 5-HT receptors. In support of that notion, experiments on NR8383 cells show a very small increase in $[Ca^{2+}]_i$ upon 5-HT (10^{-5} M) stimulation, which in mouse AM was 5-HT_{2C} receptor-dependent.

CCL2 (also called MCP-1) is a major chemokine which drives monocyte recruitment to the lung under inflammatory conditions [5] in bacterial [6] and viral [7] models of lung inflammation. CCL2 is also involved in other inflammatory disorders of the lung, including allergic airway disease, acute respiratory distress syndrome and idiopathic pulmonary fibrosis

[125]. It has been shown that CCL2 production in the rat NR8383 AM cell line can be increased by ATP, and the release from induced cells can be attenuated after chelating intracellular Ca^{2+} with BAPTA [45]. Since CCL2 is stored in cytosolic granules [126], it is possible that 5-HT promotes CCL2 release via exocytosis which is a Ca^{2+}-dependent process [43]. In addition, our results indicate that CCL2 mRNA expression starts early on after 4 h of stimulation with 5-HT [122].

CCL5 (also known as RANTES) is a chemokine which plays an active role in recruiting helper T cells, monocytes and eosinophils into inflammatory sites [127, 128]. In the lung however, CCL2 plays the predominant role in recruiting monocytes, as evidenced in the setting of viral inflammation [7]. Helper T cells play an important role in coordinating immune reaction, e.g. Th1 cells secrete interferon-γ which augments macrophage killing efficacy against ingested pathogens [129].

Neutrophil chemotaxis in enhanced by CXCL1 (also known as KC), which is produced only by activated cells, e.g. LPS-stimulated endothelial cells [130], peritoneal [131] and alveolar macrophages [132]. Interestingly, CXCL1-overexpressing mice show large neutrophil infiltrates with no signs of deleterious neutrophil activation [133]. Transgenic mice, with enhanced CXCL1 expression in the lung that are challenged with *Klebsiella pneumoniae* exhibit increased neutrophil number, improved bacterial clearance and survival [134]. The present study shows that 5-HT enhances CXCL1 expression in mouse AM *in vitro*. Enhanced infiltration of non-activated neutrophils may be beneficial for lung homeostasis, e.g. in bacterial infections. More experiments are required to clarify the effects of 5-HT on cytokine and chemokine balance in the lung *in vivo*.

Serotonin levels are elevated in inflamed tissues [75]. Platelets release 5-HT in response to many stimuli, e.g. vascular wall injury, LPS [77] and following allergen challenge [78]. Platelets have been linked to many inflammatory disorders including acute lung injury [135], asthma, cystic fibrosis, atherosclerosis and sepsis [136]. It is likely that AM are reached by and react to 5-HT under local inflammatory conditions. It can be suggested that increased release of TNFα, CCL2, and CXCL1 by AM upon 5-HT stimulation may play a role in maintaining lung microenvironment, e.g. by promoting crosstalk between AM and AEC, stimulating AM in an autocrine or paracrine fashion. Moreover, cytokines and chemokines induced by 5-HT in AM may communicate information about local inflammatory conditions to circulating monocytes, neutrophils and helper T lymphocytes, which are crucial components of the immune response.

4.3 *Conclusions*

The present study identified acute cellular effects of nicotine and 5-HT upon AM. While 5-HT rapidly induced rise in $[Ca^{2+}]_i$, nicotine did not alter the level of $[Ca^{2+}]_i$ in AM. This corroborates previous reports that nAChR subunits do not form classical ionotropic receptors in the cells of the immune system. In the presence of nicotine, they dampen Ca^{2+}-release from intracellular stores induced by stimulation with 5-HT or ATP. Since rise in $[Ca^{2+}]_i$ in AM is involved in pro-inflammatory signaling, the present study identifies the first acute receptor-mediated but atypical nicotinic effect on AM with anti-inflammatory potential. The receptor subtypes involved and intracellular signaling pathways, as identified so far, differ from that known from the nervous system and from systemic cholinergic anti-inflammation. Potentially, this allows for selective pharmacological intervention and therapeutic use.

This study identifies 5-HT as a novel modulator of AM function. We provide the first evidence for an exclusive role of the 5-HT_{2C} receptor in serotonergic AM activation, assessed by increase in $[Ca^{2+}]_i$ and changes in cytokine/chemokine profiles upon 5-HT treatment. Activation of AM by 5-HT might play an important role in communicating pro-thrombotic events in the pulmonary vasculature to resident lung immune cells. These data warrant consideration during pharmacological modulation of 5-HT metabolism and might offer an opportunity for the modification of AM function *in vivo*.

5 Summary

Alveolar macrophages (AM) hold a key position in initiating inflammatory responses in the lung by secreting TNFα and several additional cytokines and chemokines. Their activity is controlled by paracrine signals such as ATP which might either be actively release as a signaling molecule or liberated during cell death, thereby reflecting a danger signal. The present study was aimed to determine to which extend, and via which receptor, serotonin (5-HT), an amine released from activated platelets, and nicotinic acetylcholine receptors (nAChR), a receptor class that has been identified in mediating anti-inflammatory effects, also participate in paracrine modulation of AM function.

The expression of 5-HT type 2 receptors and the effects evoked by stimulation with 5-HT were investigated in mouse AM. Expression of the 5-HT receptors was analyzed by RT-PCR. Alveolar macrophages expressed receptor subtypes 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}, with the strongest and most consistent expression being noted for 5-HT_{2C} receptors. In mouse AM, 5-HT (10^{-5} M) induced a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) that was initiated by release of Ca^{2+} from intracellular stores and depended on extracellular Ca^{2+} in a sustained phase. This 5-HT-induced increase in $[Ca^{2+}]_i$ was neither observed in AM treated with the 5-HT_{2C} receptor-selective inhibitor RS102221 nor in AM derived from 5-HT_{2C} receptor-deficient mice. Alveolar macrophages stimulated with 5-HT (10^{-5} M) showed increased production of CCL2, CCL5 and TNFα as determined by a dot-blot assay. These data demonstrate the presence of functional 5-HT_{2C} receptors on AM and suggest a role of 5-HT as novel modulator of AM function. Importantly, these effects are exclusively driven by the 5-HT_{2C} receptor, thereby providing the potential for selective intervention.

In addition, the nAChR inventory of freshly isolated rat and mouse AM was determined and the cellular events evoked by stimulation with nicotine were investigated. Positive RT-PCR results in rat AM were obtained for nAChR subunits α3, α5, α9, α10, β1, and β2, with most stable expression of subunits α9, α10, β1, and β2. Mouse AM expressed nAChR subunits α9, α10, β2 and β4. Notably, mRNA coding for subunit α7, which is proposed to convey the nicotinic anti-inflammatory response of macrophages from other sources than the lung, was not detected. RT-PCR data were supported by immunohistochemistry on rat AM isolated by lavage, as well as in lung tissue sections and by Western blotting. Measurements of $[Ca^{2+}]_i$ in rat and mouse AM did not reveal any changes in response to nicotine. However, nicotine (10^{-4} M), given 2 min prior to ATP and 5-HT, significantly reduced the agonist-induced rise in $[Ca^{2+}]_i$. This nicotinic effect was further studied in rat AM, where it was blocked by α-

bungarotoxin and did not depend on the presence of extracellular calcium. These data demonstrate that AM are equipped with modulatory nAChR with properties distinct from ionotropic nAChR mediating synaptic transmission in the nervous system. Their stimulation with nicotine dampens ATP-induced Ca^{2+}-release from intracellular stores. Thus, the present study identifies the first acute receptor-mediated nicotinic effect on AM with anti-inflammatory potential.

Taken together, 5-HT, an amine released from activated platelets, and nAChR, a receptor class that has been identified in mediating anti-inflammatory effects, participate in paracrine modulation of AM function. Serotonergic stimulation of 5-HT_{2C} receptors activates AM, whereas nAChR confer an inhibitory influence upon both serotonergic and purinergic activation via pathways differing from those known from the nervous system. These data warrant consideration during pharmacological modulation of cholinergic transmission and 5-HT metabolism and might offer an opportunity to modify AM function *in vivo*.

6 Zusammenfassung

Alveolarmakrophagen (AM) nehmen eine Schlüsselstellung in Entzündungsreaktionen in der Lunge ein, die sie durch die Sekretion von TNFα sowie weiteren Cytokinen und Chemokinen einleiten. Ihre Aktivität steht unter der Kontrolle parakriner Signale, beispielsweise ATP, welches entweder aktiv sezerniert wird, oder bei Zelltod freigesetzt wird und dann ein Gefahrensignal darstellt. Die vorliegende Studie hatte das Ziel festzustellen, in welchem Ausmaß und über welchen Rezeptor Serotonin (5-HT) - ein biogenes Amin, das von aktivierten Thrombozyten freigesetzt wird - und nikotinische Azetylcholinrezeptoren (nAChR) - eine Rezeptorklasse, die anti-inflammatorische Mechanismen vermitteln kann – ebenfalls an der parakrinen Modulation von AM beteiligt sind.

Die Expression von 5-HT-Rezeptoren des Typs 2 und die Auswirkungen einer serotonergen Stimulation wurden an AM der Maus untersucht. RT-PCR zeigte die Expression der Rezeptorsubtypen 5-HT_{2A}, 5-HT_{2B} sowie 5-HT_{2C}, wobei der 5-HT_{2C}-Subtyp bei weitem am stärksten exprimiert war. In AM der Maus bewirkte 5-HT (10^{-5} M) einen Anstieg der intrazellulären Kalziumkonzentration ($[Ca^{2+}]_i$), der durch eine Freisetzung aus intrazellulären Speichern initiiert wurde und in seiner Aufrechterhaltung von extrazellulärem Kalzium abhängig war. Dieser 5-HT-bedingte $[Ca^{2+}]_i$-Anstieg war nach Gabe des 5-HT_{2C}-Rezeptorantagonisten RS102221 und in AM von 5-HT_{2C}-Rezeptor-gendefizienten Mäuse nicht zu beobachten. Ein Dot-Blot-Assay zeigte eine vermehrte Produktion von CCL2, CCL5 und TNFα durch AM nach Stimulation mit 5-HT (10^{-5} M). Diese Daten zeigen das Vorkommen funktioneller 5-HT_{2C}-Rezeptoren auf AM und implizieren, dass 5-HT ein neu identifizierter Modulator der AM-Funktion ist. Da der serotonerge Effekt ausschließlich über den 5-HT_{2C}-Rezeptor vermittelt wird, ergibt sich das Potenzial einer selektiven Intervention.

Weiterführend wurden das Inventar an nAChR von frisch isolierten AM der Maus und Ratte und die durch Nikotin hervorgerufenen zellulären Effekte untersucht. In der Ratte ergaben sich positive RT-PCR-Befunde für die nAChR-Untereinheiten α3, α5, α9, α10, β1 und β2, mit konstanter Expression der Untereinheiten α9, α10, β1 und β2. Maus-AM zeigten Expression der nAChR-Untereinheiten α9, α10, β1 und β4. Es ist hervorzuheben, dass mRNA der α7-Untereinheit, welche für die anti-inflammatorische Wirkung von Nikotin auf Makrophagen anderer Organe als der Lunge verantwortlich gemacht wird, nicht nachweisbar war. Die RT-PCR-Befunde wurden durch Immunhistochemie an lavagierten Ratten-AM und Gewebeschnitten der Lunge sowie durch Western blot gestützt. Weder Maus- noch Ratten-AM reagierten auf Nikotin mit einer direkten Änderung der $[Ca^{2+}]_i$, aber sowohl der 5-HT- als

auch der ATP-induzierte $[Ca^{2+}]_i$-Anstieg wurde durch eine vorherige (2 min) Gabe von Nikotin (10^{-4} M) signifikant abgeschwächt. Dieser Nikotineffekt wurde in der Ratte weiter analysiert. Er war unabhängig von extrazellulärem Kalzium und ließ sich durch α-Bungarotoxin hemmen. Diese Befunde zeigen, dass AM mit modulierend wirkenden nAChR ausgestattet sind, deren Eigenschaften sich von den ionotropen nAChR des Nervensystems unterschieden, die dort die cholinerge synaptische Übertragung vermitteln. In AM dämpft ihre Stimulation die ATP-induzierte Freisetzung von Kalzium aus intrazellulären Speichern. Die vorliegende Arbeit identifiziert somit den ersten akuten rezeptorvermittelten nikotinischen Effekt auf AM.

Zusammenfassend sind sowohl 5-HT - ein biogenes Amin, das von aktivierten Thrombozyten freigesetzt wird – als auch nAChR - eine Rezeptorklasse, die anti-inflammatorische Mechanismen vermitteln kann – an der parakrinen Modulation von AM beteiligt. Serotonin stimuliert AM über $5\text{-}HT_{2C}$-Rezeptoren, während nAChR über Signalwege, die sich von denen aus dem Nervensystem bekannten unterscheiden, einen inhibitorischen Einfluss auf serotonerge und purinerge AM-Aktivierung ausübt. Diese Daten sollten bei der pharmakologischen Modulation cholinerger Transmission und des 5-HT-Metabolismus berücksichtigt werden und könnten zugleich eine Möglichkeit der Modulation der AM-Aktivität *in vivo* bieten.

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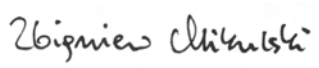
8 Declaration

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.



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Publications

1. **Mikulski, Z.**, Hartmann, P., Jositsch, G., Zasłona, Z., Lips, KS., Pfeil, U., Kurzen, H., Lohmeyer, J., Clauss, WG., Grau, V., Fronius, M., Kummer, W. (2009) Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. The manuscript is under revision in *Journal of Leukocyte Biology* as J0908-558R.

2. **Mikulski, Z.**, Zasłona, Z., Cakarova, L., Hartmann, P., Wilhelm, J., Tecott, LH., Lohmeyer, J., Kummer, W. (2009) Serotonin activates murine alveolar macrophages through 5-HT_{2C} receptors. *Manuscript in preparation.*

3. Hecker, A., **Mikulski, Z**., Lips, KS., Pfeil, U., Zakrzewicz, A., Wilker, S., Hartmann, P., Padberg, W., Wessler, I., Kummer, W., Grau, V. (2009) Pivotal Advance: Up-regulation of acetylcholine synthesis and paracrine cholinergic signalling in intravascular transplant leukocytes during rejection of rat renal allografts. *J Leuk Biol.* 86:13-22.

Oral presentations

1. **Mikulski, Z.**, Hartmann, P., Lips, K.S., Biallas, S., Pfeil, U., Grando, S.A., Grau, V., Kummer, W. (2007) Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. *102nd Annual Meeting of the Anatomische Gesellschaft, Giessen, Germany, 30 March – 2 April 2007.*

2. **Mikulski, Z.**, (2007) Nicotinic receptors on rat alveolar macrophages. *MBML annual retreat, Rauischholzhausen, Germany, 9 – 11 July 2007.*

3. **Mikulski, Z.**, (2008) Serotonin-induced intracellular calcium mobilization in mouse alveolar macrophages. *MBML annual retreat, Rauischholzhausen, Germany, 28 – 30 July 2008.*

4. **Mikulski, Z.**, Zaslona Z., Cakarova, L., Hartmann, P., von Wulffen, W., Lohmeyer, J., Kummer, W. (2009) Serotonin acutely elevates intracellular calcium concentration and augments CCL2 production in murine alveolar macrophages. *104th International Meeting of the Anatomische Gesellschaft, Antwerpen, Belgium, 27 -30 March 2009.*

5. **Mikulski, Z.** (2009) Serotonin acutely elevates intracellular calcium concentration and augments CCL2 production in murine alveolar macrophages. *MBML annual retreat, Rauischholzhausen, Germany, 27 – 29 July 2009.*

Poster presentations

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3. **Mikulski, Z.**, Zaslona, Z., von Wulffen, W., Kummer, W. (2008) Serotonin-induced rise in intracellular [Ca^{2+}] in mouse alveolar macrophages is modulated by nicotine. *Annual Congress of the American Thoracic Society, Toronto, Canada, 16-22 May 2008*. Published as abstract in *Am J Resp Crit Care Med.* 177:A621.

4. **Mikulski, Z.**, Zaslona, Z., von Wulffen, W., Kummer, W. (2008) Serotonin-induced rise in intracellular [Ca^{2+}] in mouse alveolar macrophages is modulated by nicotine. *ECCPS Symposium on Remodeling and Reverse Remodeling in the Cardiopulmonary System, Bad Nauheim, Germany, 29 June – 2 July 2008*.

5. Zaslona, Z., **Mikulski, Z.**, Cakarova, L., Hartmann, P., von Wulffen, W., Lohmeyer, J., Kummer, W. (2009) Serotonin acutely elevates intracellular calcium concentration and augments CCL2 production in murine alveolar macrophages. *World Immune Regulation Meeting III, Davos, Switzerland, 22 – 25 March 2009.* Best Poster Prize ”Late breaking abstracts”.

6. **Mikulski, Z.**, Zaslona, Z., Cakarova, L., von Wulffen, W., Kummer, W. (2009) Serotonin acutely elevates intracellular calcium concentration and augments CCL2 production in murine alveolar macrophages. *Annual Congress of the American Thoracic Society, San Diego, USA, 15-20 May 2009*. Published as abstract in *Am J Resp Crit Care Med.* 179:A1345.

Conferences attended as a non-presenting delegate

2nd International Symposium on Non-neuronal Acetylcholine, Mainz, Germany, 31 August – 2 September, 2006.

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First von Behring-Röntgen Symposium Neurotransmission: then, now and tomorrow - The lessons of Loewi, Marburg, 18-19 November 2009.

Workshops

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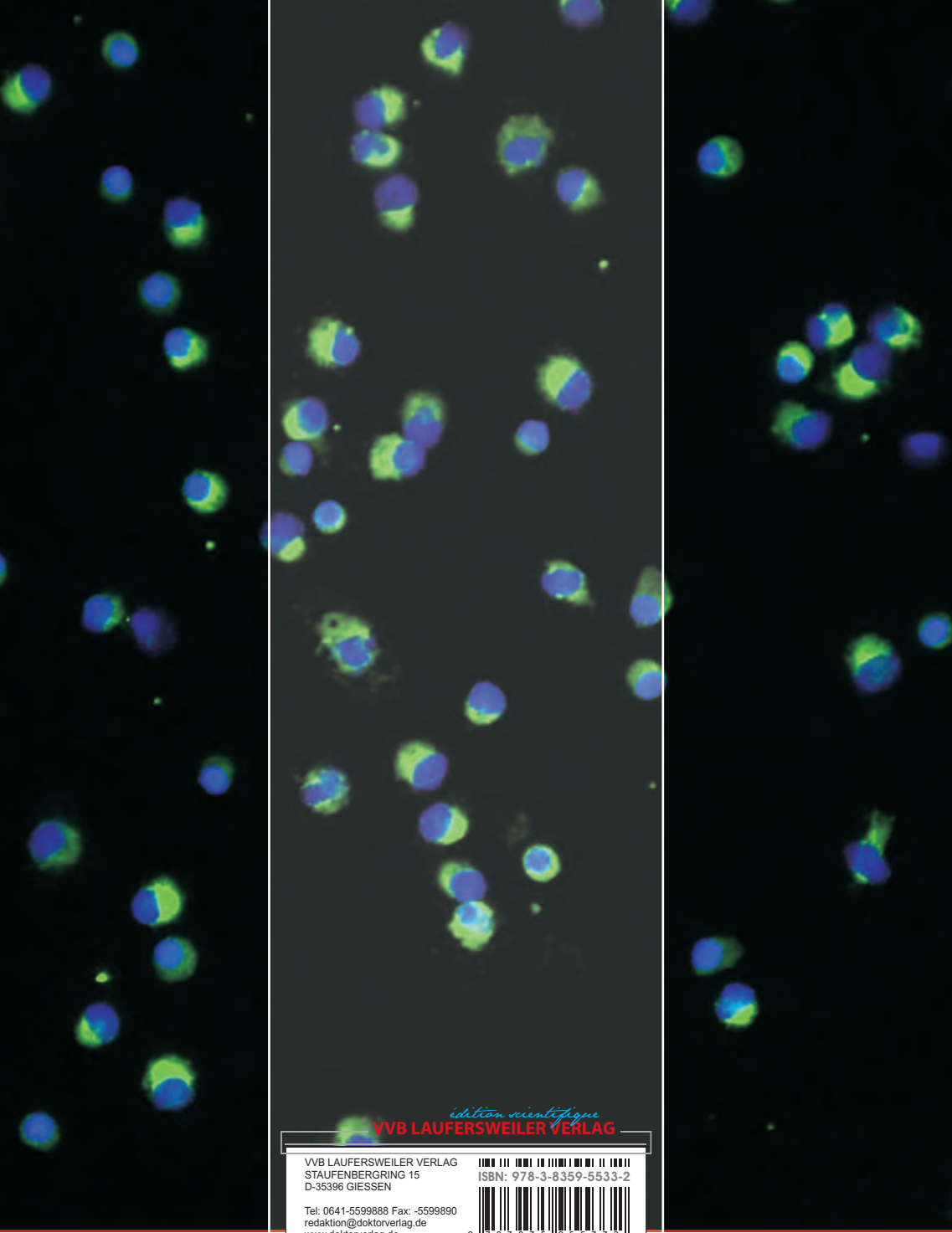

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