The physiological relevance of the 
G protein-coupled receptor P2Y$_{14}$

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UDP-sugars were identified as extracellular signaling molecules, assigning a new function to these compounds in addition to their well-defined role in intracellular substrate metabolism and storage. Previously regarded as an orphan receptor, the G protein-coupled receptor (GPCR) P2Y\textsubscript{14} (GPR105) was found to bind extracellular UDP and UDP-sugars. Little is known about the physiological functions of this GPCR. To study its physiological role a gene-deficient (KO) mouse strain expressing the bacterial LacZ reporter gene was used to monitor the physiological expression pattern of P2Y\textsubscript{14}. P2Y\textsubscript{14} is mainly expressed in pancreas and salivary glands and in subpopulations of smooth muscle cells of the gastrointestinal tract, bronchioles, blood vessels and uterus. Among other phenotypical differences KO mice showed a significantly impaired glucose tolerance following oral and intraperitoneal glucose application. An unchanged insulin tolerance points towards an altered pancreatic islet function. Transcriptome analysis of pancreatic islets showed that P2Y\textsubscript{14} deficiency significantly changed expression of components involved in insulin secretion. Insulin secretion tests revealed a reduced insulin release from P2Y\textsubscript{14}-deficient islets highlighting P2Y\textsubscript{14} as a previously unappreciated modulator of proper insulin secretion.
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<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALAT</td>
<td>alanine transaminase</td>
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<tr>
<td>ASAT</td>
<td>aspartate transaminase</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>Cch</td>
<td>carbachol</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>CNS</td>
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<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<td>COS-7</td>
<td>fibroblast-like cell line</td>
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<tr>
<td>Ct</td>
<td>threshold cycle</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMR</td>
<td>dynamic mass redistribution</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>ECMV</td>
<td>Encephalomyocarditis virus</td>
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<td>ES-cell</td>
<td>embryonic stem cell</td>
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<tr>
<td>FPKM</td>
<td>Fragments Per Kilobase of transcript per Million mapped reads</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<td>GLDH</td>
<td>glutamate dehydrogenase</td>
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<td>GLUT2</td>
<td>glucose transporter 2</td>
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<td>GO</td>
<td>gene ontology</td>
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<td>G protein-coupled receptors</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<td>HCT</td>
<td>hematocrit</td>
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<tr>
<td>HDL</td>
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<td>human embryonic kidney 293 cells</td>
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<tr>
<td>HGB</td>
<td>hemoglobin</td>
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<tr>
<td>IP₃</td>
<td>inositol (1,4,5)-trisphosphate</td>
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IRES  internal ribosome entry site
ITT   insulin tolerance test
KO    knockout
KRB   Krebs-Ringer solution
LacZ  β-galactosidase gene
LDL   low-density lipoprotein
loxP  locus of X-over P1
MCH   mean corpuscular hemoglobin
MCHC  mean corpuscular hemoglobin concentration
MCV   mean corpuscular volume
MPV   mean platelet volume
mRNA  messenger ribonucleic acid
NPY   neuropeptide Y
OD    optical density
oGTT  oral glucose tolerance test
PBS   phosphate buffered saline
PKA   protein kinase A
PLCβ  phospholipase Cβ
PLT   platelets
qPCR  quantitative real time polymerase chain reaction
RBC   red blood cell
RDW   red blood cell distribution width
RGS   regulator of G-protein signaling
RMPI  Roswell Park Memorial Institute medium
SD    standard deviation
SEM   standard error of the mean
SNP   single nucleotide polymorphism
SPF   specific pathogen free
TAG   triacylglycerol
UDP   uridine diphosphate
UTR   untranslated region
VLDL  very low-density lipoprotein
WBC   white blood cell
WT    wildtype
1 INTRODUCTION

In the regulation of virtually all physiological functions of the human organism, G protein-coupled receptors (GPCR) play a central role. GPCR are involved in the perception of environmental stimuli like light, smell or taste as well as in endocrine and neuronal signaling controlling both cognitive functions and unconscious homeostatic processes. This includes regulation of nutrient uptake, storage and utilization and energy balance, processes that are subject to great attention in metabolic disorder research. With their genes comprising 3% of the entire genome, GPCR form one of the largest protein families (1,2). These over 800 genes can be classified due to structural and physiological aspects. However, the recently used GRAFS classification system groups the receptors in following five classes using phylogenetic features: Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F), and Secretin (S) receptors (3). The most studied rhodopsin receptor class comprises about 700 GPCR, including nearly 400 odorant receptors.

Despite their activation by structurally different ligands and their high functional diversity every GPCR is shown to consist of seven transmembrane helices which are connected by three intracellular and three extracellular loops. Whereas the extracellular N-terminus is in charge of ligand binding, the intracellular C-terminus interacts with a heterotrimeric G protein that determines the downstream signaling. Upon ligand binding conformational changes in the receptor result in the exchange of GDP to GTP and dissociation of the α-subunit and the βγ-dimer of the G-protein complex. Both are able to activate further effectors (4). Usually G proteins are referred to by the isoform of their α-subunit. So far more than 20 isoforms have been identified which can be assigned to four major groups: Gs, Gi/o, Gq/11 and G12/13. Gs coupling stimulates, Gi/o coupling inhibits the adenyl cyclase activity resulting in elevation or decrease of intracellular cAMP levels, respectively. Activation of Gq/11 subunit leads to generation of second messengers diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP3) via activation of phosholipase Cβ (PLCβ) (5). G12/13 subunit mediates its signaling by stimulation of Rho guanine-nucleotide exchange factors. However, βγ-dimers as well are shown to affect the activity of ion channels, RGS proteins, and certain types of adenylyl cyclases and phospholipases C (6).

1.1 P2Y14 discovery as an orphan GPCR

Although the physiological function is known for many members of the family of GPCR and about 36% of currently approved drugs act via their activation (7), functional ligands have
not been identified yet for more than one hundred so-called orphan receptors. These receptors were predicted from DNA sequences which share sequence motifs characteristic of GPCR using homology screening techniques, such as degenerate polymerase chain reaction and low-stringency hybridization (8,9). In a classic approach, bioactive substances, that were potentially GPCR ligands, were used to identify the receptor. Cloning methods evolved in the late 1980’s and allowed to test possible ligands and tissue extracts on orphan GPCR expressed in heterologous cell systems. The serotonin 5-HT1A receptor (10) and the dopamine D2 receptor (11) were the first deorphanized GPCR. Using this so-called reverse pharmacology approach most of the identified GPCR were assigned to their endogenous agonists by academic research groups and pharmaceutical companies engaged in large-scale deorphanization efforts (9).

Thus, one of the studies discovered P2Y14 (formerly referred to as KIAA0001, VTR 15-20, GPR 105) as a GPCR. KIAA0001 was initially cloned from a human myeloid cell line and shows several attributes (e.g. seven transmembrane domains, consensus sequences for protein kinase phosphorylation, extracellular N-terminus) characteristic for GPCR (12,13). As known for diverse GPCR, the human P2Y14 gene as well shows genomic clustering with other GPCR. P2Y14 is co-localized with P2Y12, P2Y13, GPR87 and GPR171 on the long arm of the chromosome 3 (14).

1.2 P2Y14 as a member of the P2Y12-like receptor group

P2Y14 belongs to the P2Y12-like receptor group within the family of rhodopsin-like receptors. P2 receptors are activated by extracellular purine and pyrimidine nucleotides and are subdivided into two main families, the P2X ionotropic ligand-gated ion channel receptors and the P2Y metabotropic GPCR. On the basis of their amino acid sequences the eight identified P2Y receptors form two distinct subgroups. The P2Y1 group includes P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11, whereas the P2Y12 group contains the P2Y12, P2Y13 and P2Y14.

Based on structural similarity and phylogenetic aspects the P2Y12-like receptor group comprises, additional to the above mentioned P2Y12-14 receptors, the orphan receptors GPR34, GPR82, GPR87, and GPR171 (Fig. 1). Phylogenetic analyses of this receptor family show the presence of these GPCR in cartilaginous and bony fishes suggesting their existence for more than 450 million years. The high degree of structural conservation within the P2Y12-like receptor group is a sign of selective constraint on the sequences during the evolution and emphasizes the importance of this group in regulating physiological functions (14).
Based on phylogenetic and structural aspects the P2Y\textsubscript{12}-like receptor group forms a distinct subgroup within the P2Y receptors (14).

The most studied ADP receptor P2Y\textsubscript{12} plays a significant role in platelet aggregation and several antithrombotic drugs like clopidogrel, ticagrelor and prasugrel target this GPCR in order to prevent myocardial infarction and strokes. Patients with inactivating mutations in P2Y\textsubscript{12} were shown to have bleeding disorders (15,16). A number of groups characterized the role of P2Y\textsubscript{13} in cholesterol metabolism (17-19) and several ongoing studies discuss its influence in bone development (20-22). Knockout (KO) mouse studies revealed the involvement of the orphan GPR82 in energy homeostasis, and indeed, SNP variants in GPR82 could be associated with lower BMI in a human population (23). However, only little is known about the physiological relevance of the other members of the P2Y\textsubscript{12}-like receptor group.

1.3 Ligand-binding studies and G-protein coupling of P2Y\textsubscript{14}

In the past years, a number of P2Y\textsubscript{12}-like receptors were assigned to their endogenous agonists, including various substance classes like nucleotide derivates and lipids (24-27).
Thus, P2Y₁₂-like receptor family seems to have high ligand promiscuity showing broad pharmacological profiles as known for other GPCR receptor groups like trace amine-associated receptors (28,29) and MAS-related GPCR (9).

In 2000, P2Y₁₄ was deorphanized using the reverse pharmacology approach. Chambers et al. (30) screened several GPCR, expressed in a heterologous yeast cell system, against a huge library of potential GPCR agonists and found UDP-glucose to activate P2Y₁₄. This was an interesting finding, since UDP-glucose was previously only known as a glucosyl donor in glycogen biosynthesis. Several closely related sugars like UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine showed agonistic activity on this GPCR as well. These results were verified in mammalian HEK-293 cell both, transiently and stably expressing the P2Y₁₄ gene and the promiscuous G protein α₁-subunit Go₁₆ (30).

Chambers (30) as well studied the G-protein coupling of P2Y₁₄ in GTPγS radioactive assays. UDP-glucose promoted GTPγS binding to endogenous G proteins could be blocked by pre-incubation of mammalian cells with pertussis toxin, indicating that P2Y₁₄ mediates the inhibition of adenylyl cyclase via coupling to Go₁ subunits. Fricks (31), Moore (32), Ko (33) and other colleagues used a more robust experimental approach to investigate the signal transduction pathway of this GPCR. Thus, a chimeric Goᵢ₄₄ subunit was coexpressed with P2Y₁₄ to redirect the Gᵢ signaling to the PLC-β pathway following Ca²⁺ response. These experiments confirmed the preferred Gᵢ coupling of P2Y₁₄ stimulated with UDP-glucose and other UDP-activated sugars.

In more recent studies, UDP alone was controversially discussed as agonist for P2Y₁₄. No agonistic activity of UDP and other P2Y receptor nucleotide ligands was seen in GTPγS binding assays of HEK-293 cells stably expressing P2Y₁₄ (30). Scrivens et al. could not detect any effect of UDP on forskolin-stimulated cAMP levels in isolated human neutrophils and T-lymphocytes (34,35) and similar results were reported by Ko and his colleagues in COS-7 cells transiently co-expressing the human P2Y₁₄ and the Gᵢ₅ protein (33). However, using different human receptor mutants of P2Y₁₄ Ault and Broach tested antagonistic activity of UDP on UDP-glucose-mediated receptor stimulation (36). Subsequent analyses specified that the detected UDP antagonism was competitive (31). On the other hand, further studies with classic cAMP inhibition assays (37) and nonconventional methods like cellular impedance functional assays (38) described UDP as a potent agonist on human P2Y₁₄.
1.4 Physiological relevance of P2Y\textsubscript{14}

Tissue distribution is often used to anticipate a possible function of a novel protein. P2Y\textsubscript{14} mRNA expression and Northern blot analyses showed a widespread expression pattern including high expression levels in placenta, adipose tissue, stomach and intestine and moderate levels in spleen, lung, heart and different brain regions (32,39). P2Y\textsubscript{14} was also detected in immune cells, such as B- and T-lymphocytes, neutrophils, dendritic cells, astrocytes, and microglia (32,34,35,40,41). Based on the high prevalence of this GPCR in the immune system, previous studies focused on elucidating the involvement of P2Y\textsubscript{14} in immune and inflammatory responses. Indeed, expression of P2Y\textsubscript{14} was upregulated following inflammatory injury (42) and similar results were shown by \textit{in vivo} treatment with lipopolysaccharide for rat brain and spleen (13,32). P2Y\textsubscript{14} was reported to mediate mast cell degranulation (43) and dendritic cell maturation by intracellular Ca\textsuperscript{2+} mobilization (41). Further, Kook et al. (44) described protective effects of P2Y\textsubscript{14} signaling pathway in response to radiation.

Functional studies regarding the high expression levels in the gastrointestinal tract revealed an influence of P2Y\textsubscript{14} in gastric motility function, however Bassil et al. demonstrated that some effects of UDP-glucose were independent of P2Y\textsubscript{14} signaling (45).

A very recent study identified P2Y\textsubscript{14} as one of the 293 GPCR expressed in human pancreatic islets (46). It is known that GPCR play an important role in regulation of hormone secretion from pancreatic islets. Depending on their presence in one of the major islet cells (\(\alpha\)-, \(\beta\)-, or \(\delta\)-cells) the certain GPCR have a different impact on the release of glucagon, insulin or somatostatin (46). Interestingly, only a few GPCR targeting drugs (e.g. GLP-1 receptor agonist exenatide) are approved for the therapy of type 2 diabetes (46). For several nucleotide receptors (e.g. P2Y\textsubscript{1}, P2Y\textsubscript{6}, P2Y\textsubscript{13}) the influence on the secretion of insulin and glucagon has been shown (46). However, the particular cell type within the pancreatic islet that expresses P2Y\textsubscript{14} and its relevance in hormone secretion has not been studied yet.

Considering the fact, that GPCR are related to several endocrine disorders and even malignant diseases (47) and that GPCR are highly relevant as drug targets, previous P2Y\textsubscript{14} studies showed enormous potential of this GPCR for future therapeutic applications, but the physiological relevance of P2Y\textsubscript{14} still remains to be defined in more detail.
2 OBJECTIVES

The UDP-glucose receptor P2Y$_{14}$ is a member of the P2Y$_{12}$-like receptor group. Although UDP-sugars and UDP were identified as endogenous agonists of this GPCR the literature is controversial regarding their physiological role. Further, the physiology of P2Y$_{14}$ is not understood in detail. Therefore, this study aimed at the physiological characterization of P2Y$_{14}$ function \textit{in vivo}. Because high expression was found in the gastrointestinal system, the main focus of this study was directed on exploring a possible role of P2Y$_{14}$ in the regulation of nutrient uptake and energy metabolism. Taking advantage of a P2Y$_{14}$-deficient and LacZ-reporter mouse model the following questions were addressed:

1. Where is P2Y$_{14}$ expressed in mice?
2. Does P2Y$_{14}$ deficiency have a major impact on mouse ontogenesis?
3. Does P2Y$_{14}$ influence smooth muscle function?
4. Does and how does P2Y$_{14}$ affect the regulation of glucose homeostasis?
3 EXPERIMENTAL PROCEDURES

3.1 Materials

If not stated otherwise, all standard substances were obtained from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), and C. Roth GmbH (Karlsruhe, Germany). Cell culture material was purchased from Sarstedt (Nümbrecht, Germany).

3.2 Mice

All mice were maintained in a specific pathogen-free barrier facility on a 12-h light/12-h dark cycle with ad libitum access to water and food. Experiments were performed according to the accepted standards of animal care and were approved by the respective regional government agency of the State of Saxony, Germany (T27/11; T46/12; T07/13).

3.2.1 Generation of P2Y14-deficient mice

P2Y14-KO mice were generated at TAKEDA Ltd. (Cambridge, United Kingdom) by targeted disruption of the mouse P2Y14 locus and ES-cell blastocyst injection. Thus, most part of the coding region of P2Y14 was exchanged by a β-galactosidase cassette harbouring an internal ribosome entry site (IRES) followed by a loxP-floxed neomycin cassette (Fig. 2).

Figure 2 Generation of a P2Y14-deficient mouse strain and P2Y14 qPCR design.

P2Y14-deficient mice were generated at TAKEDA Ltd.. The coding sequence was partly replaced by an ECMV-IRES / β-galactosidase cassette followed by a loxP-floxed neomycin selection cassette. The neomycin cassette was removed by breeding with EIIa-Cre mice and successful removal was verified by PCR. For qPCR a primer pair matching to exons 1 and 2 was designed. Genotyping of mice was performed as described under 3.2.2 with primers indicated as: for, rev and β-gal s.
Neomycin-resistant ES cells were screened for homologous recombination. Positive ES cell clones were injected into blastocysts. Chimeric offspring was fertile and crossed into a 129S6 background. These mice were crossed with EIIa-Cre mice (C57Bl/6 strain) to remove the neomycin cassette. Correct deletion was verified by PCR and sequencing of the locus. The resulting KO animals were bred with 129S6 animals and all studies were done on a mixed C57BL/6 x 129S6 background with predominance of 129S6. For experiments WT and KO littermates from intercrossed WT and KO parents were used.

3.2.2 Genotyping

Genotyping of mice was carried out by PCR with the following primers:
- β-gal sense: 5’-AGAAGGCACATGGCTGAATATCGA-3’
- forward: 5’-AGCTGCCGGACGAAGGAGACCCTGCTC-3’
- reverse: 5’-GGTTTTTGGAAACCTCTAGGTCATTCTG-3’

in two separate PCR reactions (Fig. 2): forward/reverse to amplify WT allele and β-gal sense/reverse to amplify KO allele. The PCR conditions were 95°C for 3 minutes followed by 35 cycles with 45 sec 95°C, 60°C 30 sec and 72°C for 1 min and a final amplification step of 72°C for 10 min. Amplification of the WT allele and the KO allele resulted in 180-bp and 400-bp fragments, respectively. PCR products were separated via gel electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination.

3.3 Basic characterization of P2Y14-deficient mice

3.3.1 Morphology

Three-month-old mice were inspected for differences in breeding, growth, weight and morphology. Heterozygous females were bred with heterozygous males to document the litter genotype distribution. Body and tail lengths of mice were measured. Animals were monitored for morphological abnormalities during coat, teeth and limb development. Organs were removed and weighed.

3.3.2 Quantitative expression analysis

Several tissues were removed from 3 WT and 3 KO mice and RNA was isolated using TRIZOL (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer’s instructions. RNA quantity was measured with a spectrophotometer (Nanodrop ND 1000, NanoDrop products, Wilmington, USA) and RNA quality was controlled by gel electrophoresis. For quantitative real time PCR (qPCR) 1 μg of total RNA was reversely
transcribed (Superscript II RT, Invitrogen, Karlsruhe, Germany) using oligo-dT primers. qPCR was performed by Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to manufacturer’s instructions. Primers were designed for the intron-flanking exons 1 and 2 sequences (Fig. 2) to exclude genomic contaminations (sense: 5’-GAAGCCAGACGTGAAGGAGTT-3’; antisense: 5’-CAGGAATCTCAAAGGCAAGCT-3’) resulting in a 156-bp product. qPCR was performed with the MX 3000P instrument (Agilent Technologies GmbH, Boeblingen, Germany) using the following protocol: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 15 s at 95 °C and 30 s at 60 °C. A product dissociation curve was recorded to verify the presence of a single amplicon. Threshold cycle (Ct) values were determined during the exponential increase of the product in the PCR. After normalization to the house keeping gene β2-microglobulin calculated ∆Ct values were used to determine the relative expression of P2Y14.

3.3.3 LacZ reporter gene assay

Tissue samples of several organs were prepared from WT and KO mice, covered in Tissue-Tek® (Sakura, Torrance, CA, USA) and snap frozen in liquid nitrogen. The organs were cryosectioned (10 µm) and fixed in acetone-methanol (1:1) solution. Sections were incubated with X-Gal staining solution (500 µg/ml X-gal in 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 150 mM NaCl and 2 mM MgCl₂ diluted in 0.45 mM HEPES buffer, pH 7.4) at 30 °C overnight to detect the expression of the LacZ reporter gene via β-galactosidase activity. Following, nuclear fast red staining was performed.

3.3.4 Laboratory chemistry / histology

Serum levels of electrolytes, metabolites, enzymes and hormones were analyzed in three-month-old WT and KO mice according to the guidelines of the German Society of Clinical Chemistry and Laboratory Medicine, using a Hitachi PPE-Modular analyzer (Roche Diagnostics, Mannheim, Germany). Blood cell counting from EDTA blood samples was performed automatically (ScilVet abc; Scil corporation, Viernheim, Germany). Histological slices (5 µm) were prepared from organs being fixed in 4% formaldehyde solution and embedded in paraffin wax. Slices were stained with H&E.

3.3.5 Behavioral tests

The open field test was performed as reported previously (48) using automated measuring technology (TSE Systems, Bad Homburg, Germany). Activity of mice was recorded for a period of 5 min. In a hot plate test (Hot Plate 602001, TSE) the elapsed time until the first
reaction of the mice to the heat stimulus (52°C) was recorded. As endpoints shaking or licking of one of the hind paws or jumping off the analgesia meter were used.

### 3.3.6 Assessment of hemodynamic parameters

Blood pressure and pulse were determined by non-invasive tail-cuff method using the BP-2000 Blood Pressure Analysis System™ (Visitech Systems, USA). Following manufacturer’s instructions, mice were trained to the procedure each day for at least 5 days prior to the actual experiment. After the training period 10 measurements per day of each mouse on 5 consecutive days were performed. Systolic blood pressure, diastolic blood pressure, mean arterial pressure, and pulse were calculated from daily experiments for each mouse and mean out of 10 animals per group was determined.

### 3.4 Smooth muscle function

#### 3.4.1 Gastrointestinal transit experiments

A gastrointestinal emptying test was performed by oral application of 200 µl dye-labeled non-absorbable liquid dextran blue (20 mg/ml) to mice. Faeces were collected separately for each mouse hourly for 8 hours, vortexed with 300 µl phosphate buffered saline (PBS) and centrifuged at 13,000 rpm for 10 min. The amount of hourly excreted dextran blue was measured photometrical at 620 nm in supernatants. Mice that showed no excretion for at least 2 hours were excluded from the experiment. The sum of totally excreted dextran blue was set 100% and the relative expulsion of dextran blue was compared hourly between the WT and KO mice.

#### 3.4.2 Gastrointestinal contractility studies

For contractility studies mice were sacrificed by cervical dislocation. The intestine from mice was harvested and placed in ice-cold Krebs-Ringer buffer (KRB, 117 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, 11.5 mM glucose, pH 7.4). One cm long equal segments of intestine were placed in 50-ml organ baths (37 °C) containing KRB solution and continuously gassed with 95% O₂ and 5% CO₂. To record the contractile activity, each intestine segment was connected to an isometric force transducer (MLT0201 Force Transducer; ADInstrumends GmbH, Spechbach, Germany). The basal tension was set to 10 mN. Intestine segments that did not show spontaneous activity were excluded from the experiment. After a 30 min equilibration phase the effects of 100 µM UDP-glucose and 100 µM carbachol (positive control) were tested. Each stimulation period
was monitored for at least 10 min. Intestine segments were washed three times after each stimulation period. Several parameters like mean muscle tension, average tension maximum and minimum, average amplitude, rate of contractions and the area under the curve were analyzed using Lab Chart software (ADInstruments). Data were normalized to recorded spontaneous activity and expressed in percentage.

3.4.3 Airway responsiveness

Lung resistance and dynamic compliance were measured by invasive plethysmography (emka TECHNOLOGIES, Paris, France) in response to inhaled UDP-glucose and methacholine (Sigma-Aldrich). Female mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine; Bayer Leverkusen, Germany), intubated and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline lung resistance, dynamic compliance, and responses to aerosolized saline (0.9% NaCl) were measured first, followed by responses to 100 µM aerosolized UDP-glucose and increasing doses (10 to 80 mg/ml) of aerosolized methacholine (49).

3.5 Metabolic studies

One-month-old mice were kept on standard (Ssniff M-Z: 5% sugar, 4.5% raw fat, 34% starch, 22% raw protein) or western (Ssniff EF R/M TD88137: 32.8% sugar, 21.2% raw fat, 14.5% starch, 17.1% raw protein) type diet (Ssniff GmbH, Soest, Germany) for 3 months. During this period body-weight development was monitored weekly. All metabolic tests (see below) were performed in the same set of animals at the age of 4 months at the interval of one week.

3.5.1 Glucose and insulin tolerance tests

For glucose tolerance tests mice on a standard or a western type diet had been fasted overnight (15 h) and blood glucose levels were measured from tail vein blood before (0 min), 20, 40, 60 and 120 min after oral application (80 mg) or intraperitoneal injection (2 mg/g body weight) of glucose. For insulin tolerance tests blood glucose levels were measured before (0 min) and 15, 30 and 60 min after intraperitoneal injection of human insulin (0.75 U/kg body weight). Blood glucose levels were determined by using an automated blood glucose meter (FreestyleLite, Abbott Diabetes Care Ltd., Oxon, UK).
3.5.2 Insulin secretion tests

For insulin secretion tests mice fasted overnight were injected intraperitoneally with glucose (2 mg/g body weight) and blood samples were taken before (0 min), 20, 40 and 60 min after injection. Serum insulin concentrations were measured using an Ultra Sensitive Mouse Insulin Elisa Kit (Crystal Chem, Inc., Downers Grove, IL, USA) in accordance to manufacturer’s instructions.

3.5.3 Morphometric analysis of pancreatic tissue

Pancreata from 5 WT and 5 KO mice fed with western type diet were fixed in 4% formaldehyde and embedded in paraffin wax. Two longitudinal sections (4 µm) were generated every 100 µm of tissue. Each time, the first section was stained with H&E, the second immunostained for insulin with the avidin-biotin complex technique using anti-insulin monoclonal rabbit antibody (100 µg/ml, Cell Signalling, Millipore, Germany) and goat anti-rabbit secondary antibody (7.5 mg/ml, Vector Laboraties, Biozol Diagnostica, Germany). Both sections were photographed with a Zeiss Axioimager Z1 microscope (Carl Zeiss Jena GmbH, Jena, Germany). Ten H&E stained sections per mouse were analyzed for number and area size of pancreatic islets using NIH ImageJ software (http://rsbweb.nih.gov/ij/). For determination of β-cell area per islet the insulin-positive area of at least 50 islets per mouse was calculated using NIH ImageJ software and related to the total islet area of the investigated slides.

3.6 Molecular and functional characterization of pancreatic islets

3.6.1 Islet isolation

Pancreatic islets were isolated as previously described (50-52). Briefly, mice were sacrificed by cervical dislocation and the pancreas was inflated by intraductal injection of ice-cold DMEM media containing 0.2 mg/ml (1.08 Wünsch Units) Liberase TL Research Grade (Roche Diagnostics GmbH, Mannheim, Germany). Distended pancreas was digested by shaking in a 37 °C water bath for 17 minutes. Isolated islets were washed and picked under a stereomicroscope and incubated overnight at 37°C and 5% CO₂ in RPMI-1640 media containing fetal bovine serum for insulin secretion studies.

3.6.2 RNA-sequencing of pancreatic islets

Total RNA from isolated pancreatic islets (10 male mice per genotype) was extracted by using TRIZOL (Sigma-Aldrich) according to the manufacturer’s instructions. RNA quantity
was measured by using a spectrophotometer (Nanodrop ND 1000). The RNA quality of all samples was further analyzed on the Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip. cDNA libraries were generated using TruSeq RNA Sample Preparation Kits v2 (Illumina, San Diego, CA, USA) according to the manufacturer’s protocol. Indexed islet libraries of good quality were pooled and used for sequencing on two flow cell lanes on Illumina HiScanSQ System. To confirm base calling correctness the raw data passed a quality check using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). It revealed low PHRED-scale scores at the 3’ end of the 101 bp long reverse reads. These reads were trimmed to a size of 80 bp. Then, reads were assigned to the individual animals using the ligated adapter indices. Further, the samples proceeded filtering to trim the adapter sequences and remove low-quality reads which were shorter than 60 bp or contained more than five bases with a quality score below 15 (PHRED-scale). The paired-end reads were mapped to the reference mouse genome (July 2007 NCBI37/mm9) with Ensembl v66 annotations using Tophat 1.3.3. (53), which aligns reads using Bowtie (version 0.9.9). Mitochondrial reads and reads which did not map uniquely to a genome position were excluded. FPKM (fragments per kilobase of transcript per million mapped reads) values were calculated using Cufflinks 1.3.0 (53,54). Analysis of differential expression was performed using DESeq software package (55). Only genes that were expressed at least in 10 animals were considered for analysis. Functional enrichment analysis of differentially expressed genes (P < 0.05) was determined using ToppGene Suite (56).

3.6.3 Insulin release experiments from isolated islets

For dynamic insulin secretion studies groups of 50 pancreatic islets were placed in a perifusion chamber and continuously perifused with KRB containing (all in mM) 115 NaCl, 20 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.56 CaCl2, 10 HEPES pH 7.4, 0.1% bovine serum albumin, and 2.8 glucose for over 30 min (flow: 1 ml/min). After this equilibration phase islets were perifused for 30 min with low glucose (2.8 mM) to determine the basal insulin release and then challenged for 30 min with 16.7 mM glucose or 16.7 mM glucose plus 100 µM UDP-glucose. Samples of perifusate were collected every minute and insulin concentration was determined at indicated time points by Insulin AlphaLISA Kit (Perkin Elmer, Rodgau, Germany) following manufacturer’s instructions.

For static incubation studies groups of 5 islets were placed in a 96-well plate. After a 30-minute equilibration period in 200 µl KRB solution containing 2.8 mM glucose, pancreatic islets were stimulated with KRB solution containing either 2.8 mM or 16.7 mM glucose or
100 µM UDP-glucose in the presence of 16.7 mM glucose for 30 minutes. The supernatants were then collected for measurement of secreted insulin and islets were lysed in acid-ethanol to extract the residual islet insulin (50,51). Insulin concentrations were determined by Insulin AlphaLISA Kit (Perkin Elmer) following manufacturer’s instructions.

### 3.7 Cell culture, transfection and functional assays

For yeast experiments, the haploid *Saccharomyces cerevisiae* yeast strain MPY578t5 (provided by Dr. Mark Pausch) was used for the expression of receptor constructs. Cells were transfected with plasmid DNA using electroporation as described (57). For expression in mammalian cells, COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified 5% CO₂ incubator. Transient transfection experiments of COS-7 cells with receptor constructs for cAMP inhibition measurements and for direct cAMP measurements (co-transfected with the chimeric G protein Gαsi5) were essentially performed as described previously (58). For measurements of dynamic mass redistribution (DMR) (59) HEK293 cells were grown in DMEM/F12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO₂ incubator and transiently transfected with the receptor constructs and split onto fibronectin-coated 384-well plates one day prior the assay. DMR measurements were performed with the Epic system (Corning, NY, US).

For all transfection experiments the mouse and human P2Y₁₄ coding sequence attached to an N-terminal hemagglutinin (HA) epitope and a C-terminal FLAG epitope was cloned into the mammalian expression vector pcDps. Additionally, for yeast experiments the mouse receptor was introduced into the yeast vector p416GPD. All control receptor plasmids were cloned with the described strategy and verified by Sanger sequencing.

### 3.8 Statistical analyses and graphics

If not stated otherwise statistical analyses were performed using Student’s t-test and graphics were designed using the GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA).
4 RESULTS

4.1 Basic characterization of P2Y_{14}-deficient mice

To identify phenotypes related to the lack of P2Y_{14} function, this GPCR gene was removed by targeted deletion and replacement with a LacZ reporter gene (Fig. 2) in a mouse strain. Mice were kept in a SPF unit and under these conditions P2Y_{14} deficiency is compatible with life. The P2Y_{14}-deficient mouse offspring was vital and fertile. No obvious differences in genotype distribution, breeding, growth, gross morphology and histology were detected on primary inspection compared to WT mice. However, comparison of the organ weights revealed a significantly higher spleen weight in P2Y_{14}-KO mice (Table 1).

Table 1 Body and organ weights of P2Y_{14}-deficient in comparison to WT mice.

<table>
<thead>
<tr>
<th>Organ</th>
<th>WT (n=19)</th>
<th>KO (n=28)</th>
<th>WT (n=24)</th>
<th>KO (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>body weight (g)</td>
<td>25.08 ± 2.25</td>
<td>25.88 ± 1.96</td>
<td>20.9 ± 1.1</td>
<td>22.0 ± 1.34</td>
</tr>
<tr>
<td>brain (g)</td>
<td>0.46 ± 0.03</td>
<td>0.46 ± 0.02</td>
<td>0.46 ± 0.01</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>heart (g)</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>lung (g)</td>
<td>0.13 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>liver (g)</td>
<td>1.1 ± 0.12</td>
<td>1.15 ± 0.12</td>
<td>0.82 ± 0.09</td>
<td>0.86 ± 0.11</td>
</tr>
<tr>
<td>spleen (g)</td>
<td>0.07 ± 0.02</td>
<td>0.11 ± 0.03***</td>
<td>0.07 ± 0.01</td>
<td>0.09 ± 0.02***</td>
</tr>
<tr>
<td>left kidney (g)</td>
<td>0.2 ± 0.03</td>
<td>0.22 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>right kidney (g)</td>
<td>0.2 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
</tbody>
</table>

Moreover, the analysis of hemodynamic parameters displayed higher blood pressure and heart rate in mice lacking P2Y_{14} (Table 2).

Table 2 Higher blood pressure and pulse in P2Y_{14}-deficient mice.

Blood pressure and pulse were determined by non-invasive tail-cuff method using the BP-2000 Blood Pressure Analysis SystemTM (Visitech Systems, USA). Following manufacturer’s instructions, mice were trained to the procedure each day for at least 5 days prior to the actual experiment. After the training period 10 measurements per day of each mouse on 5 consecutive days were performed. Systolic blood pressure, diastolic blood pressure, mean arterial pressure, and pulse were calculated from daily experiments for each mouse and mean out of 10 animals per group was determined. Data shown as mean ± SEM. ** P < 0.01, *** P < 0.001; Student’s t-test.

<table>
<thead>
<tr>
<th>parameter</th>
<th>WT (n=10)</th>
<th>KO (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean arterial pressure (mmHg)</td>
<td>84.3 ± 1.0</td>
<td>93.8 ± 1.4***</td>
</tr>
<tr>
<td>systolic pressure (mmHg)</td>
<td>121.4 ± 1.0</td>
<td>129.8 ± 1.9**</td>
</tr>
<tr>
<td>diastolic pressure (mmHg)</td>
<td>64.9 ± 1.3</td>
<td>75.7 ± 2.1***</td>
</tr>
<tr>
<td>pulse (min^{-1})</td>
<td>584 ± 15</td>
<td>654 ± 12**</td>
</tr>
</tbody>
</table>
In an open field test KO mice were significantly less active than the WT mice spending less
time on locomotion and showing a reduced velocity (Table 3). However, no distinct
behavioral phenotype could be detected on initial characterization. There was no difference
between WT and KO mice in the hot plate test (Table 3).

**Table 3 Behavioral parameters.**

Open field test: 3-month-old male mice were tested in the setup. Recording and analysis was
performed automatically. For hot plate test animals were set on a 52 °C heated plate and time until
visible signs of pain were seen was measured. The table shows main parameters as mean ± SD. *P <
0.05; **P < 0.01; Student's t-test

<table>
<thead>
<tr>
<th>OPENFIELD TEST</th>
<th>WT (n=16)</th>
<th>KO (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity (s)</td>
<td>102.3 ± 38.3</td>
<td>74.1 ± 34.2*</td>
</tr>
<tr>
<td>Total activity (%)</td>
<td>34.1 ± 12.8</td>
<td>24.7 ± 11.4*</td>
</tr>
<tr>
<td>Counts</td>
<td>1068 ± 532</td>
<td>775 ± 386</td>
</tr>
<tr>
<td>Velocity (cm s⁻¹)</td>
<td>18.9 ± 5.4</td>
<td>15.1 ± 1.7*</td>
</tr>
<tr>
<td>Distance (m)</td>
<td>23.1 ± 10.9</td>
<td>14.3 ± 6.7**</td>
</tr>
<tr>
<td>Distance edges (m)</td>
<td>21.8 ± 9.8</td>
<td>13.2 ± 7.1</td>
</tr>
<tr>
<td>Distance center (m)</td>
<td>1.3 ± 1.8</td>
<td>1.1 ± 1.9</td>
</tr>
<tr>
<td>Distance corners (m)</td>
<td>12.6 ± 4.6</td>
<td>8.9 ± 4.2*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HOT PLATE TEST</th>
<th>WT (n=9)</th>
<th>KO (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>time until signs of pain (s)</td>
<td>24 ± 5.5</td>
<td>21.6 ± 5</td>
</tr>
</tbody>
</table>

Whole blood and serum samples from adult mice were analyzed by an automatic
hemocytometer and at the Institute of Laboratory Medicine (Medical Faculty, University of
Leipzig). Particular differences in hematological and laboratory parameters were not
consistent and rather gender-specific (Tables 4 and 5).
**Table 4 Hematology.**

Whole blood samples from three-month-old mice were analyzed by an automatic hemocytometer (ScilVet ABC; scil animal care company GmbH, Viernheim, Germany). Results are given as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.

<table>
<thead>
<tr>
<th>standard diet</th>
<th>male</th>
<th>female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=15)</td>
<td>KO (n=11)</td>
</tr>
<tr>
<td>WBC (10^³ / mm³)</td>
<td>9.4 ± 2</td>
<td>10.4 ± 3.5</td>
</tr>
<tr>
<td>RBC (10^6 / mm³)</td>
<td>8.4 ± 0.7</td>
<td>8.1 ± 1.3</td>
</tr>
<tr>
<td>HGB (g / dl)</td>
<td>16.9 ± 1.1</td>
<td>16 ± 2.2</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>47.5 ± 4.2</td>
<td>44.6 ± 4.4</td>
</tr>
<tr>
<td>PLT (10^³ / mm³)</td>
<td>1175 ± 172</td>
<td>1248.5 ± 260.8</td>
</tr>
<tr>
<td>MCV (µm³)</td>
<td>56.7 ± 2.1</td>
<td>55.9 ± 8.7</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>20.3 ± 1.9</td>
<td>19.9 ± 2</td>
</tr>
<tr>
<td>MCHC (g / dl)</td>
<td>35.8 ± 3.3</td>
<td>35.9 ± 4.1</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>13.3 ± 0.7</td>
<td>14.9 ± 0.6 ***</td>
</tr>
<tr>
<td>MPV (µm³)</td>
<td>5.2 ± 0.3</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>LYMPHOCYTES (%)</td>
<td>72.6 ± 4.7</td>
<td>70.3 ± 5.6</td>
</tr>
<tr>
<td>MONOCYTES (%)</td>
<td>6.2 ± 0.6</td>
<td>6.7 ± 0.9</td>
</tr>
<tr>
<td>GRANULOCYTES (%)</td>
<td>21.2 ± 4.6</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>LYMPHOCYTES (10³ / mm³)</td>
<td>6.8 ± 1.6</td>
<td>7.2 ± 2.3</td>
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<tr>
<td>MONOCYTES (10³ / mm³)</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>GRANULOCYTES (10³ / mm³)</td>
<td>2.1 ± 0.5</td>
<td>2.5 ± 1.3</td>
</tr>
<tr>
<td>Measure</td>
<td>WT (n=15)</td>
<td>KO (n=15)</td>
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<tr>
<td>-------------------------</td>
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<tr>
<td><strong>Western Diet</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC (10^3 / mm³)</td>
<td>9.6 ± 1.8</td>
<td>8.3 ± 1.8</td>
</tr>
<tr>
<td>RBC (10^6 / mm³)</td>
<td>11.3 ± 0.7</td>
<td>11.4 ± 1.3</td>
</tr>
<tr>
<td>HGB (g / dl)</td>
<td>18.7 ± 1</td>
<td>18.3 ± 1.5</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>64 ± 3.1</td>
<td>61.6 ± 6</td>
</tr>
<tr>
<td>PLT (10^3 / mm³)</td>
<td>1031.3 ± 205.1</td>
<td>985 ± 162.4</td>
</tr>
<tr>
<td>MCV (μm³)</td>
<td>57.2 ± 2.9</td>
<td>54.1 ± 2.3**</td>
</tr>
<tr>
<td>MCHC (g / dl)</td>
<td>29 ± 1.1</td>
<td>29.8 ± 1.0 *</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>14.1 ± 2.2</td>
<td>14.6 ± 1.2</td>
</tr>
<tr>
<td>MPV (μm³)</td>
<td>5.2 ± 0.4</td>
<td>5 ± 0.3</td>
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<tr>
<td>LYMPHOCYTES (%)</td>
<td>72 ± 6.2</td>
<td>71.4 ± 6.5</td>
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<tr>
<td>MONOCYTES (%)</td>
<td>5.9 ± 0.9</td>
<td>4.8 ± 0.7 **</td>
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<td>GRANULOCYTES (%)</td>
<td>22.1 ± 6</td>
<td>23.8 ± 6.6</td>
</tr>
<tr>
<td>LYMPHOCYTES (10^3 / mm³)</td>
<td>6.9 ± 1.6</td>
<td>5.9 ± 1.6</td>
</tr>
<tr>
<td>MONOCYTES (10^3 / mm³)</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1 ***</td>
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<tr>
<td>GRANULOCYTES (10^3 / mm³)</td>
<td>2.2 ± 0.6</td>
<td>2 ± 0.6</td>
</tr>
</tbody>
</table>
Four-month-old male and female WT and KO mice were subjected to analysis for cholesterol and TAG levels in lipoprotein fractions after a western diet and clinical chemistry parameters. Results are given as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.

<table>
<thead>
<tr>
<th>standard diet</th>
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<tbody>
<tr>
<td></td>
<td>male</td>
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<td>ALAT (µkat/l)</td>
<td>0.46 ± 0.15</td>
<td>below detection</td>
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<td>ALBUMIN (g/l)</td>
<td>29.46 ± 3.17</td>
<td>27.77 ± 5.67</td>
<td>33.07 ± 4.66</td>
<td>34.05 ± 2.96</td>
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<td>ALCALINE PHOSPHATASE (µkat/l)</td>
<td>0.6 ± 0.06</td>
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<td>0.77 ± 0.17</td>
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<td>ASAT (µkat/l)</td>
<td>6.01 ± 3.77</td>
<td>1.88 ± 1.88**</td>
<td>1.53 ± 0.38</td>
<td>1.6 ± 0.41</td>
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<td>CALCIUM (mmol/l)</td>
<td>2.11 ± 0.17</td>
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<td>2.71 ± 0.32</td>
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<td>2.66 ± 0.53</td>
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<td>GLDH (µkat/l)</td>
<td>0.27 ± 0.25</td>
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<td>0.23 ± 0.08</td>
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<td>UREA (mmol/l)</td>
<td>6.97 ± 1.26</td>
<td>8.8 ± 1.51**</td>
<td>4.32 ± 1.03</td>
<td>3.4 ± 0.60**</td>
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<td>LIPASE (µkat/l)</td>
<td>0.68 ± 0.21</td>
<td>0.49 ± 0.16*</td>
<td>0.47 ± 0.14</td>
<td>0.46 ± 0.11</td>
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<td>MAGNESIUM (mmol/l)</td>
<td>0.96 ± 0.18</td>
<td>1.12 ± 0.13*</td>
<td>1.29 ± 0.24</td>
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<td>PROTEIN (g/l)</td>
<td>49.23 ± 3.73</td>
<td>47.7 ± 3.63</td>
<td>53.28 ± 6.03</td>
<td>54.03 ± 3.14</td>
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<td>PHOSPHATE (mmol/l)</td>
<td>2.05 ± 0.37</td>
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<td>1.84 ± 0.24</td>
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<td>ALAT (µkat/l)</td>
<td>0.67 ± 0.36</td>
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<td>0.35 ± 0.03</td>
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<td>23.89 ± 3.05</td>
<td>23.97 ± 4.2</td>
<td>30.83 ± 3</td>
<td>30.31 ± 3.41</td>
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<td>ALCALINE PHOSPHATASE (µkat/l)</td>
<td>0.57 ± 0.13</td>
<td>0.62 ± 0.11</td>
<td>0.78 ± 0.24</td>
<td>0.63 ± 0.12</td>
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<td>ASAT (µkat/l)</td>
<td>6.12 ± 3.5</td>
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<td>CALCIUM (mmol/l)</td>
<td>1.9 ± 0.19</td>
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<td>CHOLESTEROL (µkat/l)</td>
<td>89.44 ± 11.23</td>
<td>86.45 ± 21.08</td>
<td>154.12 ± 12.47</td>
<td>152.62 ± 17.96</td>
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<td>GLDH (µkat/l)</td>
<td>3.99 ± 0.55</td>
<td>4.13 ± 0.86</td>
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<td>LIPASE (µkat/l)</td>
<td>0.62 ± 0.31</td>
<td>0.99 ± 0.96</td>
<td>0.56 ± 0.19</td>
<td>0.59 ± 0.37</td>
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<td>MAGNesium (mmol/l)</td>
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<td>1.19 ± 0.28</td>
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<td>PROTEIN (g/l)</td>
<td>47.23 ± 4.35</td>
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<td>58.46 ± 3.42</td>
<td>57.63 ± 3.49</td>
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<td>2.19 ± 0.36</td>
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<td>2.67 ± 0.56</td>
<td>2.51 ± 0.38</td>
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<td>TRIGLYCERIDES (mmol/l)</td>
<td>0.97 ± 0.34</td>
<td>1.22 ± 0.58</td>
<td>0.94 ± 0.16</td>
<td>1.03 ± 0.59</td>
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<td>KO (n=15)</td>
<td>male</td>
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<td>HDL cholesterol (mmol/l)</td>
<td>4.49 ± 0.5</td>
<td>4.52 ± 0.99</td>
<td>3.13 ± 0.54</td>
<td>3.23 ± 0.52</td>
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<td>HDL triacylglycerides (mmol/l)</td>
<td>0.76 ± 0.07</td>
<td>0.75 ± 0.1</td>
<td>0.31 ± 0.05</td>
<td>0.38 ± 0.14</td>
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<td>LDL cholesterol (mmol/l)</td>
<td>1.86 ± 0.22</td>
<td>1.76 ± 0.31</td>
<td>1.87 ± 0.3</td>
<td>1.58 ± 0.17**</td>
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<td>LDL triacylglycerides (mmol/l)</td>
<td>1.46 ± 0.29</td>
<td>1.32 ± 0.19</td>
<td>0.55 ± 0.1</td>
<td>0.49 ± 0.11</td>
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<td>VLDL cholesterol (mmol/l)</td>
<td>0.22 ± 0.05</td>
<td>0.23 ± 0.04</td>
<td>0.31 ± 0.08</td>
<td>0.28 ± 0.09</td>
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<td>VLDL triacylglycerides (mmol/l)</td>
<td>1.16 ± 0.31</td>
<td>1.25 ± 0.29</td>
<td>0.44 ± 0.13</td>
<td>0.37 ± 0.12</td>
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<td>total cholesterol (mmol/l)</td>
<td>6.57 ± 0.72</td>
<td>6.52 ± 1.3</td>
<td>5.31 ± 0.79</td>
<td>5.09 ± 0.62</td>
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4.2 \( \text{P2Y}_{14} \) expression in mouse tissues

Because the primary inspection did not reveal any prominent differences between WT and KO mice I next took advantage of the introduced LacZ reporter construct and monitored \( \beta \)-galactosidase activity in \( \text{P2Y}_{14} \)-deficient mouse tissues to examine the \( \text{P2Y}_{14} \) expression at the cellular level. Whereas WT control tissues showed no specific staining, \( \beta \)-galactosidase-positive staining was found in uterus, bronchioles, blood vessels, pancreas, salivary glands (Fig. 3), and immune cells.

![Figure 3 Detection of bacterial LacZ reporter gene via \( \beta \)-galactosidase activity in cryosections of selected organs.](image)

To monitor the expression of \( \text{P2Y}_{14} \) \textit{in vivo} at the cellular level a transgenic KO mouse was generated by replacement of the \( \text{P2Y}_{14} \)-coding region with an expression cassette harboring an internal ribosome entry site and the bacterial LacZ reporter gene under control of the endogenous \( \text{P2Y}_{14} \) promoter. Sites of LacZ expression were located in cryosections using X-Gal, and counterstained with nuclear fast red. Whereas no \( \beta \)-galactosidase activity was detected in WT mice, KO mice showed intensive \( \beta \)-galactosidase staining in several smooth muscle or contractile cell-containing organs. A indicates arterial vessel, V indicates venous blood vessel.
Interestingly, staining in the GI tract was positive only in a subpopulation of smooth muscle cells (Fig. 4A/B). In all parts of the GI tract smooth muscle cells of the thin layer of the *muscularis mucosae* were β-galactosidase positive (Fig. 4A/B). From the ileum to the rectum a subpopulation of smooth muscle cells within the circular smooth muscle layer showed positive staining (Fig. 4A/B). In the rectum also the longitudinal smooth muscle layer displayed intensive β-galactosidase activity (Fig. 4A/B).

![Figure 4 P2Y₁₄ is expressed in a subpopulation of smooth muscle cells in the gastrointestinal tract.](image)

Expression of P2Y₁₄ was monitored *in vivo* using a LacZ reporter gene (see 3.3.3). (A) KO mice showed intensive β-galactosidase staining in a subpopulation of smooth muscle cells throughout the GI tract. (B) The expression of P2Y₁₄ in certain muscle layers of the GI tract is schematically given.

Co-staining of c-kit, which is a marker of Cajal cells and mast cells in the GI tract (60,61), revealed no obvious overlap with the β-galactosidase-positive structures (data not shown). In contrast the β-galactosidase staining was partly reminiscent of the reported divergent reactivity of intestinal smooth muscle cells with characteristic smooth muscle cell markers (i.e. alpha-smooth muscle actin (ASMA), gamma-smooth muscle actin (GSMA)) (62). Interestingly, cells next to the pancreatic acini (Fig. 5A-C) and myoepithelial cells of the salivary glands (Fig. 3) were stained. Further, few cells within the islet (Fig. 5 B/D) were β-galactosidase-positive. However, the distribution pattern of these cells did not completely correspond with one of the known major cell types within the islet of Langerhans.
Figure 5 Detection of bacterial LacZ reporter gene in cryosections of pancreas.

(A/B) Expression of LacZ reporter gene in exocrine and endocrine parts of the pancreas. The enlarged picture details show the expression in the acini (B/C) and in cells of the islet (D).

One should keep in mind that P2Y\textsubscript{14} promoter-driven β-galactosidase-expression was monitored in KO mice and may reflect transcript over- or even non-physiological expression due to P2Y\textsubscript{14} deficiency. To address this issue, expression of the 5’UTR region of WT and KO P2Y\textsubscript{14} gene transcripts, which were structurally similar in both WT and KO animals, was measured by qPCR in a number of tissues (Fig. 6). As presented in Fig. 6B, ΔCt values of transcript expression showed a high correlation between WT and KO in all tissues ($r^2 = 0.9058$).
Figure 6 P2Y\textsubscript{14} transcript and P2Y\textsubscript{14}-promoter-driven LacZ gene expression in mouse tissues.

Total RNA was extracted from several tissues from WT and P2Y\textsubscript{14}-KO mice (n=3) and mRNA levels of either P2Y\textsubscript{14} or LacZ transcripts were determined by SYBR Green qPCR analysis. Expression data are shown as \(\Delta C_t\) values normalized to the house keeping gene \(\beta_2\)-microglobulin. (A) qPCR analysis shows a widespread receptor distribution with high expression levels in pancreas, salivary glands, brain and parts of the GI tract and lowest expression in liver. Data shown as mean ± SEM. (B) Expression of bacterial LacZ reporter gene in KO mice correlates \((r^2=0.9058)\) with the expression of P2Y\textsubscript{14} in WT mice. Dashed line indicates the 95% confidence interval.

Both data sets revealed that P2Y\textsubscript{14} is widely expressed in mouse tissues with high expression levels in pancreas, salivary glands, brain, lung and parts of the gastrointestinal tract and lowest expression in liver (Fig. 6).

4.3 Characterization of P2Y\textsubscript{14} function in smooth muscles

4.3.1 Delayed GI passage in P2Y\textsubscript{14}-deficient mice

To study the consequences of P2Y\textsubscript{14} deficiency in the GI tract, different segments of the intestine were placed in an organ bath and the contractile activity was recorded. Duodenal segments and segments of ileum and colon displayed regular basal activity showing no differences regarding mean muscle tension, amplitude or frequency of contractions between the genotypes. Following carbachol and electric stimulation all intestine segments of WT and KO mice showed an increase of contractile activity in a similar manner. Because no effect of
UDP-glucose (up to 1 mM) on basal and carbachol-stimulated and electric-stimulated contraction of isolated small intestine and colon was found (data not shown), I speculated that the contribution of P2Y<sub>14</sub> is more distinct and may present its significance only when the entire GI passage is observed. Therefore, a dye-labeled dextran was applied to mice orally and the excretion in faeces was measured hourly. On average three hours after the oral application of dextran blue the mice begun to excrete the dye. In comparison to WT animals the expulsion of dextran blue was significantly delayed in KO mice (Fig. 7). Interestingly, more KO animals (5 KO vs. 1 WT) had to be excluded from analysis according to the exclusion criterion (see 3.4.1).

**Figure 7 P2Y<sub>14</sub>-deficient mice showed a delayed gastrointestinal emptying.**

A dye-labeled dextran was applied to mice orally and the excretion in faeces was measured hourly. The excretion of blue dextran is shown relative to the total excreted blue dextran after 8 hours. Five KO mice but only one WT animal had to be excluded from analysis according to the exclusion criterion (see 3.4.1). OD<sub>620 nm (∆ 8h)</sub> = 0.8770 ± 0.0630 for WT and OD<sub>620 nm (∆ 8h)</sub> = 0.5636 ± 0.0731 for KO mice were set to 100%, respectively. Data shown as mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t-test

**4.3.2 Altered airway responsiveness in P2Y<sub>14</sub>-deficient mice**

P2Y<sub>14</sub>-reporter gene expression was also found in smooth muscle cells of bronchioles (Fig. 3). To dissect P2Y<sub>14</sub> function in respiratory tract invasive plethysmography experiments were performed testing airway resistance and dynamic compliance in intubated and mechanically ventilated mice. Aerosolized UDP-glucose had no effect on respiratory functions tested (Fig. 8). Both WT and KO mice displayed adequate response to the cholinergic agent methacholine.
(Fig. 8A). However, KO mice showed significantly higher dynamic compliance in response to increasing inhaled doses of methacholine (Fig. 8B).

Figure 8 Airway responsiveness is reduced in P2Y$_{14}$-deficient mice.

Lung resistance (A) and dynamic compliance (B) were measured by invasive plethysmography (emka TECHNOLOGIES) in response to inhaled UDP-glucose and increasing doses of methacholine. Data shown as mean ± SD. Baseline values (set to 100%) were: lung resistance 1.28 ± 0.41 mmHg x s/ml for WT mice and 1.11 ± 0.34 mmHg x s/ml for KO mice, dynamic compliance 0.84 ± 0.56 ml/mmHg for WT and 0.72 ± 0.43 ml/mmHg for KO mice; * P < 0.05; Student’s t-test. For statistic analysis of the entire methacholine test a paired two-tailed t-test (marked by a long line) was performed (P = 0.0105 for dynamic compliance).

4.4 P2Y$_{14}$ deficiency alters metabolic functions

4.4.1 P2Y$_{14}$ deficiency results in reduced glucose tolerance

The initial phenotype screen also included basic metabolic tests. Following oral glucose application KO mice showed significantly higher transient blood glucose levels (Fig. 9A). To differentiate whether the enteral glucose resorption was increased in KO mice due to the prolonged gastrointestinal emptying (see Fig. 7), intraperitoneal glucose tolerance tests were performed. Blood glucose levels were also increased in KO mice following intraperitoneal glucose injection (Fig. 9B) excluding differences in enteral glucose resorption. In order to evaluate the insulin sensitivity in KO mice insulin tolerance tests were performed. Both genotypes showed no alterations in the decline of blood glucose levels following intraperitoneal insulin injection (Fig. 10A), suggesting unchanged insulin sensitivity in KO mice. Serum insulin levels were analyzed during intraperitoneal glucose tolerance tests. Here, KO mice tended to have lower serum insulin levels after intraperitoneal glucose application (Fig. 10C).
4.4.2 Challenging the metabolic phenotype with a western type diet

To study whether high caloric nutrition can aggravate the phenotype found in KO mice, 4-week-old mice were kept on a western type diet for 3 months. WT and KO mice gained significantly body weight under the western type diet. But whereas there were no alterations in body weight development between WT and KO mice kept on standard diet, KO mice fed the western diet gained significantly less weight (KO: 178 ± 15% vs. WT: 196 ± 23%, P < 0.05). However, detailed analysis of serum cholesterol and TAG levels in all lipoprotein fractions revealed no differences between WT and KO mice fed a western type diet (see 4.1 Table 5). Interestingly, glucose tolerances following oral and intraperitoneal glucose application were significantly reduced in KO mice kept under western type diet (Fig. 9C/D).

Figure 9 P2Y14-deficient mice showed reduced glucose tolerance.
Glucose tolerance tests were performed either by oral application (A, C) of 80 mg glucose or by intraperitoneal (B, D) glucose injection (2 mg/g body weight) to overnight fasted male mice kept under standard (A, B) or western (C, D) type diet. Blood glucose levels shown as mean ± SD. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t-test

Next, insulin tolerance tests were performed to evaluate whether the higher glucose levels observed in KO mice in the oGTT were because of altered insulin sensitivity. There were no differences in the decline of blood glucose levels following intraperitoneal insulin injection
between WT and KO mice kept on western type diet (Fig. 10B), although both genotypes showed lower insulin sensitivity as a result of higher body weight due to high caloric nutrition. As insulin sensitivity did not account for altered oGTT in KO mice serum insulin levels in WT and KO mice during intraperitoneal glucose tolerance tests were analyzed. Interestingly, after 12 weeks of western type diet glucose-stimulated insulin secretion of KO mice was significantly lower 40 and 60 min after intraperitoneal glucose injection compared to WT mice (Fig. 10D).

Figure 10 P2Y14-deficient mice showed diet-dependent lower serum insulin levels after glucose administration.

First, blood glucose levels were measured in an insulin tolerance test (ITT) after intraperitoneal injection of insulin (0.75 U/kg body weight) in male mice kept under standard (A) and western (B) type diet. Blood glucose levels before insulin application (set to 100%) were 4.9 ± 0.6 mmol/l for WT and 5.8 ± 1.0 mmol/l for KO mice fed with standard diet, and 5.6 ± 0.7 mmol/l for WT and 6.1 ± 0.9 mmol/l for KO mice kept under western type diet. Data shown as mean ± SD. * P < 0.05; Student’s t-test.

Then, serum insulin levels were determined before (0 min) and 20, 40 and 60 min after intraperitoneal glucose injection (2 mg/g of body weight) in male mice kept under standard (C) and western type (D) diet. Data shown as mean ± SEM. ** P < 0.01; Student’s t-test
4.5 Normal architecture of pancreatic islets

As the highest expression of P2Y$_{14}$ was noticed in pancreas (Figs. 5 and 6) and the functional data pointed towards a lower pancreatic insulin secretion after oral und intraperitoneal glucose administration, morphometric analyses in H&E and insulin immunohistochemistry stained pancreatic slices in P2Y$_{14}$-KO mice were performed. Neither the number nor the size of the pancreatic islets differed between WT and KO mice (Fig. 11A/C). Also, the insulin immunopositive areas were similar in both genotypes, suggesting no gross morphological abnormalities in islet development (Fig. 11B/D).

![Figure 11 Normal architecture of pancreatic islets in P2Y$_{14}$-deficient mice.](image)

(A) Representative H&E stained pancreatic section used for morphometric analyses. (B) Insulin immunohistochemistry of representative pancreatic islets. (C) The number and area of islets in 10 serial slices of pancreatic tissue were determined using NIH ImageJ Software. The number of islets was 141 ± 17 in WT and 157 ± 11 in KO mice. (D) β-cell mass was determined as ratio of insulin positive area to total pancreatic islet area. Data shown as mean ± SD.
4.6 Components involved in insulin release are differentially expressed in P2Y₁₄-deficient islets

Since P2Y₁₄-deficient islets did not show obvious morphological differences I screened for those at a molecular level. Thus, mRNA expression in isolated pancreatic islets from WT and KO mice was analyzed in a transcriptome-wide approach using RNA-sequencing. Approximately 13 million reads per animal were analyzed. The quality of the expression data was confirmed by verifying the presence of selected islet-specific transcripts. As expected, insulin, glucagon, somatostatin and SUR1/Kir6.2 transcripts were highly expressed in islets of both, WT and KO mice (Table 6).

In line with the data from immunohistochemistry there was no difference in insulin mRNA expression levels between WT and KO (Table 6). The expression of P2Y₁₄ gene was significantly reduced in KO mice (Table 6), but one should note that P2Y₁₄ transcripts in the KO islets represented mRNA sequences of the 5'UTR region and not of the coding region (see Fig. 2).

815 genes were found differentially expressed between WT and KO mice (329 transcripts downregulated, 486 transcripts upregulated, P < 0.05). Functional enrichment analysis of differentially expressed genes disclosed several GO (Gene Ontology) categories related to protein synthesis, signaling pathways and hormone secretion (Table 7).
Table 6 Expression of selected transcripts related to insulin release in P2Y14-deficient and WT mice.

Analysis of differential expression in pancreatic islets was performed using DESeq software package.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Ensembl ID Mouse</th>
<th>Description</th>
<th>normalized counts (DESeq)</th>
<th>normalized counts (DESeq)</th>
<th>p-value</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>WT</td>
<td>KO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2yr14</td>
<td>ENSMUSG00000036381</td>
<td>UDP-glucose receptor</td>
<td>150.669 ± 67.367</td>
<td>51.233 ± 31.351</td>
<td>1.55E-05</td>
</tr>
<tr>
<td>Ins1</td>
<td>ENSMUSG00000035804</td>
<td>Insulin 1</td>
<td>1152011.035 ± 292272.566</td>
<td>1259275.220 ± 367446.561</td>
<td>0.39634</td>
</tr>
<tr>
<td>Ins2</td>
<td>ENSMUSG00000000215</td>
<td>Insulin 2</td>
<td>23707662.257 ± 624362.404</td>
<td>26673072.360 ± 754468.324</td>
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</tr>
<tr>
<td>Gcg</td>
<td>ENSMUSG00000000394</td>
<td>Glucagon</td>
<td>1573442.614 ± 332358.640</td>
<td>1118086.770 ± 428209.260</td>
<td>0.00935</td>
</tr>
<tr>
<td>Sst</td>
<td>ENSMUSG00000004366</td>
<td>Somatostatin</td>
<td>109603.517 ± 26948.775</td>
<td>78143.829 ± 26039.779</td>
<td>0.03064</td>
</tr>
<tr>
<td>Insr</td>
<td>ENSMUSG00000005534</td>
<td>Insulin receptor</td>
<td>787.091 ± 93.101</td>
<td>918.837 ± 249.758</td>
<td>0.25710</td>
</tr>
<tr>
<td>Insrr</td>
<td>ENSMUSG00000005640</td>
<td>Insulin receptor-related receptor</td>
<td>15144.613 ± 3513.097</td>
<td>16533.489 ± 3935.146</td>
<td>0.55844</td>
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<tr>
<td>Gcgr</td>
<td>ENSMUSG000000025127</td>
<td>Glucagon receptor</td>
<td>1664.742 ± 428.258</td>
<td>1519.357 ± 316.953</td>
<td>0.48272</td>
</tr>
<tr>
<td>Sstr1</td>
<td>ENSMUSG000000035431</td>
<td>Somatostatin receptor 1</td>
<td>42.117 ± 32.920</td>
<td>15.753 ± 9.191</td>
<td>0.03221</td>
</tr>
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<td>Sstr2</td>
<td>ENSMUSG000000047904</td>
<td>Somatostatin receptor 2</td>
<td>184.474 ± 79.801</td>
<td>117.072 ± 66.365</td>
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</tr>
<tr>
<td>Sstr3</td>
<td>ENSMUSG000000044933</td>
<td>Somatostatin receptor 3</td>
<td>2081.031 ± 553.613</td>
<td>2084.433 ± 394.202</td>
<td>0.92098</td>
</tr>
<tr>
<td>Sstr4</td>
<td>ENSMUSG000000037014</td>
<td>Somatostatin receptor 4</td>
<td>3.725 ± 4.102</td>
<td>1.836 ± 3.349</td>
<td>0.83452</td>
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<tr>
<td>Sstr5</td>
<td>ENSMUSG000000050824</td>
<td>Somatostatin receptor 5</td>
<td>1.714 ± 3.783</td>
<td>0.841 ± 1.995</td>
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<tr>
<td>Slc2a2</td>
<td>ENSMUSG00000027690</td>
<td>GLUT2</td>
<td>13545.200 ± 4388.043</td>
<td>9864.047 ± 2396.638</td>
<td>0.02151</td>
</tr>
<tr>
<td>Gck</td>
<td>ENSMUSG000000041798</td>
<td>Glucokinase</td>
<td>5233.313 ± 2382.235</td>
<td>4290.637 ± 1140.332</td>
<td>0.47089</td>
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<tr>
<td>Abcc8</td>
<td>ENSMUSG000000040136</td>
<td>SUR1</td>
<td>35996.663 ± 7296.922</td>
<td>33147.884 ± 7820.323</td>
<td>0.65933</td>
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<td>Kcnj11</td>
<td>ENSMUSG000000070561</td>
<td>Kir6.2</td>
<td>1395.498 ± 181.626</td>
<td>1448.442 ± 401.472</td>
<td>0.87691</td>
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<tr>
<td>Pdx1</td>
<td>ENSMUSG00000029644</td>
<td>pancreatic and</td>
<td>1375.043 ± 385.507</td>
<td>1145.584 ± 120.464</td>
<td>0.16201</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Description</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>p-value</td>
</tr>
<tr>
<td>--------</td>
<td>------------------</td>
<td>--------------------------------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>Caena1c</td>
<td>ENSMUSG00000051331</td>
<td>duodenal homeobox 1</td>
<td>4416.180 ± 1642.112</td>
<td>3020.178 ± 1114.107</td>
<td>0.03284</td>
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<tr>
<td>Caena1d</td>
<td>ENSMUSG00000015968</td>
<td>L-type calcium channel α1-subunits</td>
<td>6319.115 ± 1502.032</td>
<td>6014.105 ± 1966.976</td>
<td>0.54487</td>
</tr>
<tr>
<td>Caena1f</td>
<td>ENSMUSG00000031142</td>
<td></td>
<td>126.956 ± 106.971</td>
<td>85.304 ± 35.759</td>
<td>0.36549</td>
</tr>
<tr>
<td>Caena1s</td>
<td>ENSMUSG00000026407</td>
<td></td>
<td>2.895 ± 6.173</td>
<td>5.758 ± 12.204</td>
<td>0.19040</td>
</tr>
<tr>
<td>Caena1g</td>
<td>ENSMUSG00000020866</td>
<td>T-type calcium channel α1-subunits</td>
<td>942.481 ± 437.464</td>
<td>772.241 ± 262.283</td>
<td>0.42725</td>
</tr>
<tr>
<td>Caena1h</td>
<td>ENSMUSG00000024112</td>
<td></td>
<td>978.104 ± 393.856</td>
<td>615.766 ± 174.645</td>
<td>0.04259</td>
</tr>
<tr>
<td>Caena1i</td>
<td>ENSMUSG00000022416</td>
<td></td>
<td>91.118 ± 64.381</td>
<td>65.418 ± 41.993</td>
<td>0.32531</td>
</tr>
<tr>
<td>Caena2d1</td>
<td>ENSMUSG00000040118</td>
<td>Voltage-dependent calcium channel α2δ-subunits</td>
<td>10097.878 ± 2300.350</td>
<td>9583.156 ± 2186.810</td>
<td>0.54478</td>
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<tr>
<td>Caena2d2</td>
<td>ENSMUSG00000010066</td>
<td></td>
<td>711.329 ± 348.331</td>
<td>586.156 ± 217.327</td>
<td>0.41999</td>
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<tr>
<td>Caena2d3</td>
<td>ENSMUSG00000021991</td>
<td></td>
<td>126.052 ± 22.894</td>
<td>89.065 ± 31.253</td>
<td>0.07272</td>
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<tr>
<td>Caena2d4</td>
<td>ENSMUSG00000041460</td>
<td></td>
<td>30.534 ± 13.830</td>
<td>32.242 ± 32.546</td>
<td>0.50999</td>
</tr>
<tr>
<td>Caecn1</td>
<td>ENSMUSG00000020882</td>
<td>Voltage-dependent calcium channel β-subunits</td>
<td>1236.722 ± 312.148</td>
<td>1214.727 ± 354.465</td>
<td>0.63333</td>
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<tr>
<td>Caecn2</td>
<td>ENSMUSG00000057914</td>
<td></td>
<td>1900.381 ± 463.540</td>
<td>1567.767 ± 289.201</td>
<td>0.14638</td>
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<tr>
<td>Caecn3</td>
<td>ENSMUSG00000003532</td>
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<td>2214.024 ± 387.968</td>
<td>2105.138 ± 420.606</td>
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<tr>
<td>Caecn4</td>
<td>ENSMUSG000000017412</td>
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<td>112.675 ± 41.338</td>
<td>116.486 ± 37.496</td>
<td>0.53251</td>
</tr>
<tr>
<td>Caecn1</td>
<td>ENSMUSG00000020722</td>
<td>Voltage-dependent calcium channel γ-subunits</td>
<td>0 ± 0</td>
<td>0.171 ± 0.541</td>
<td>0.75784</td>
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<tr>
<td>Caecn2</td>
<td>ENSMUSG00000019146</td>
<td></td>
<td>7.166 ± 5.535</td>
<td>3.537 ± 2.860</td>
<td>0.27770</td>
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<tr>
<td>Caecn3</td>
<td>ENSMUSG00000066189</td>
<td></td>
<td>0.978 ± 2.122</td>
<td>3.992 ± 4.077</td>
<td>0.09313</td>
</tr>
<tr>
<td>Caecn4</td>
<td>ENSMUSG00000020723</td>
<td>Voltage-dependent calcium channel γ-subunits</td>
<td>102.566 ± 48.997</td>
<td>111.682 ± 58.461</td>
<td>0.57746</td>
</tr>
<tr>
<td>Caecn5</td>
<td>ENSMUSG00000040373</td>
<td></td>
<td>1.717 ± 3.288</td>
<td>1.543 ± 2.674</td>
<td>0.88201</td>
</tr>
<tr>
<td>Caecn6</td>
<td>ENSMUSG00000078815</td>
<td></td>
<td>1.238 ± 3.915</td>
<td>1.699 ± 2.654</td>
<td>0.91835</td>
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<tr>
<td>Caecn7</td>
<td>ENSMUSG00000069806</td>
<td></td>
<td>17.747 ± 13.035</td>
<td>22.773 ± 19.587</td>
<td>0.56912</td>
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<tr>
<td>Caecn8</td>
<td>ENSMUSG00000053395</td>
<td></td>
<td>0.373 ± 1.179</td>
<td>0 ± 0</td>
<td>0.68948</td>
</tr>
</tbody>
</table>
Table 7 Functional enrichment analysis of differentially expressed genes in P2Y₁₄-deficient compared to WT mice.

<table>
<thead>
<tr>
<th>ID</th>
<th>Biological Process</th>
<th>Genes</th>
<th>(upregulated in KO)</th>
<th>p-value</th>
</tr>
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<tr>
<td>GO:0003735</td>
<td>structural constituent of ribosome</td>
<td>Rps8, Rps12, Mrp12, Rps3, Rpl36a, Rplp0, Rpl22, Rps27a, Rps15a, Rps27l, Mrp11, Mrp120, Mrp23, Mrp15, Rpl6, Rpl11, Rpl12, Rpl17, Mrp113, Mrp133, Mrp124</td>
<td>0.0000104</td>
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<tr>
<td>GO:0042886</td>
<td>amide transport</td>
<td>Fam132a, Ras110b, Bad, Ltbp4, Hla-drb1, Upk3a, Phpt1</td>
<td>0.0056743</td>
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<tr>
<td>GO:0002790</td>
<td>peptide secretion</td>
<td>Fam132a, Ras110b, Bad, Ltbp4, Hla-drb1, Phpt1</td>
<td>0.0092096</td>
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</tr>
<tr>
<td>GO:0006415</td>
<td>translational termination</td>
<td>Rps8, Rps12, Rps3, Rpl36a, Rpl0, Rpl12, Rps271, Rps15a, Ict1, Rpl6, Rpl11, Rpl12, Rpl17</td>
<td>0.0079669</td>
<td></td>
</tr>
<tr>
<td>GO:0043624</td>
<td>cellular protein complex disassembly</td>
<td>Rps8, Rps12, Rps3, Stm2, Rpl36a, Rpl0, Rpl22, Rps27a, Rps15a, Capza1, Ict1, Rpl6, Rpl11, Rpl12, Rpl17</td>
<td>0.0121003</td>
<td></td>
</tr>
<tr>
<td>GO:0007218</td>
<td>neuropeptide signaling pathway</td>
<td>Nmur1, Galr1, Hertr2, Emr1</td>
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<tr>
<td>GO:0043241</td>
<td>protein complex disassembly</td>
<td>Rps8, Rps12, Rps3, Stm2, Rpl36a, Rpl0, Rpl22, Rps27a, Rps15a, Capza1, Ict1, Rpl6, Rpl11, Rpl12, Rpl17</td>
<td>0.0186688</td>
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<td>GO:0030072</td>
<td>peptide hormone secretion</td>
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<td>GO:0015833</td>
<td>peptide transport</td>
<td>Fam132a, Ras110b, Bad, Ltbp4, Hla-drb1, Phpt1</td>
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<tr>
<td>GO:0010817</td>
<td>regulation of hormone levels</td>
<td></td>
<td></td>
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<td>------------</td>
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</tr>
<tr>
<td></td>
<td>Adh4, Adh1, Adora1, Cacna1h, Slc2a2, Glp1r, Gal, Gcg, Cacna1c, Ffar1, Hsdrb2, Pde1c, Rbp4, Itp r1, Mafa, Edn3, Ghrl, Stxbp5l, Gpr119, Cartpt, Enpep</td>
<td>Fam132a, Rasl10b, Bad, Ltbp4, Tg, Ngf, Sult1e1, Hla-drb1, Lrat, Phpt1, Reln</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0.0548341
Since serum insulin levels were reduced in KO mice I mainly focused on genes known to be related to glucose sensing, insulin exocytosis and its modulation. Selected components involved in insulin release and their changes in transcript levels are schematically given in Figure 12.

Glucose uptake through the glucose transporter 2 (GLUT2) is a first key step in regulation of insulin release (63) (Fig. 12). The expression of GLUT2 (Slc2a2) was significantly downregulated in KO.

The major trigger of insulin vesicle exocytosis is an increase in intracellular Ca\(^{2+}\) (63) and cAMP and protein kinase A (PKA) activity are known to amplify insulin exocytosis (64). In the classical model of GPCR-modulated insulin release, G_q (Ca\(^{2+}\)) and G_s (cAMP) activating receptors promote insulin release, whereas G_i/o protein-coupled receptors reduce insulin.
release by inhibiting adenylyl cyclases and reducing cAMP levels in β-cells. Indeed, several components involved in receptor- and ion channel-controlled intracellular Ca\(^{2+}\) levels were downregulated in islets of KO mice, among them the subunits CACNA1C and CACNA1H of the voltage-dependent Ca\(^{2+}\) channels (VDCC), the endoplasmic reticulum IP\(_3\) receptor (ITPR1), cAMP-degrading phosphodiesterases PDE1C and PDE4D (65), several GPCR known to modulate insulin release such as the free-fatty acid receptor FFAR1 (66), calcium-sensing receptor (CASR), GLP1 receptor (GLP1R), GPR119, somatostatin receptors SSTR1 and SSTR2 (and their agonist somatostatin) (46,67).

4.7 Reduced insulin release from pancreatic islets of P2Y\(_{14}\)-deficient mice

It is almost impossible to functionally analyze all components found to be differentially expressed between WT und KO at both, their individual level and their signaling network level. Therefore, I studied insulin secretion, as one integral islet function, in perifused and static treated islets in vitro.

Although intracellular insulin content was similar in lysed islets isolated from WT and KO mice (Fig. 13A), the insulin release following stimulation with low (2.8 mM) and high glucose (16.7 mM) during a 30 min incubation was significantly reduced in islets lacking P2Y\(_{14}\) (Fig. 13B/C). To study the kinetics of insulin release, isolated islets were placed in a perifusion chamber and continuously perfused with KRB solution containing different glucose concentrations. The initial insulin release peak and the sustained insulin release were significantly reduced in KO islets stimulated with a high glucose concentration (16.7 mM) (Fig. 13D). Stimulation of the isolated pancreatic islets with UDP-glucose alone and in presence of 16.7 mM glucose did not significantly reduce or increase insulin release (Fig. 13B-D).
Figure 13 Impaired insulin secretion of isolated pancreatic islets of P2Y_{14}-deficient mice.

For cumulative insulin measurements (A-C), pancreatic islets were isolated from WT and KO male mice and incubated 30 min with KRB solution containing 2.8 mM glucose, 16.7 mM glucose or 16.7 mM glucose plus 100 µM UDP-glucose (n=5-7). Insulin levels of lysed islets (A), in the supernatants (B) and the ratio of both (C) are shown. For kinetic insulin measurements (D), pancreatic islets were isolated from WT and KO male mice and continuously perfused with KRB solution containing 2.8 mM glucose, 16.7 mM glucose, or 16.7 mM glucose plus 100 µM UDP-glucose. Insulin levels shown as mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t-test

4.8 Functional characterization of mouse and human P2Y_{14}

Because UDP-glucose showed no physiological activity on WT mouse tissues in several assays (see 4.3.1, 4.3.2, 4.7), the agonistic properties of UDP and UDP-glucose were reevaluated using several different in vitro approaches.

First, a heterologous yeast expression system was used because it lacks any nucleotide receptors. Here, GPCR activation is linked to cell growth via a chimeric G protein. The mouse
ADP receptor P2Y\textsubscript{12} served as positive control and showed a robust cell growth upon MeS-ADP stimulation whereas UDP and UDP-glucose had no effect on P2Y\textsubscript{12} (Fig. 14). When expressed in this yeast cell system the mouse P2Y\textsubscript{14} displayed a high constitutive activity compared to P2Y\textsubscript{12} (Fig. 14A). Both, UDP-glucose and UDP, were able to slightly increase yeast growth (Fig. 14A) suggesting some agonistic activity at P2Y\textsubscript{14}.

Since results from this artificial system are not always transferable to mammalian cell expression systems (68) the mouse and the human P2Y\textsubscript{14} cotransfected with a chimeric G\textsubscript{α}_{si5} protein were tested in mammalian cells using direct cAMP measurements. This chimeric G protein directs G\textsubscript{i}-coupled receptors to the G\textsubscript{i}/adenylyl cyclase pathway. Mock transfected cells were used as negative control. The positive controls (mouse Y2 and human muscarinic M2 receptors) displayed adequate response to their agonists neuropeptide Y (NPY) and carbachol (Cch), respectively (Table 8). The human and mouse P2Y\textsubscript{14} showed increased basal activity but only an insignificant increase of cAMP levels (Table 8) upon stimulation with UDP or UDP-glucose.

**Table 8 Functional measurements of mouse and human P2Y\textsubscript{14} receptors.**

Mouse and human P2Y\textsubscript{14} constructs and two other G\textsubscript{i}-coupled receptors as controls were transfected for cAMP inhibition and direct cAMP measurements (via a chimeric G\textsubscript{α}_{si5} protein) as described in 3.8. For inhibition of cAMP, transfected COS-7 cells were stimulated either with 5 \( \mu \text{M} \) forskolin (FSK) or together with the indicated agonists for 15 minutes. Forskolin-stimulated values of each receptor were set 100\% and % of inhibition due to the agonist stimulation was calculated for each receptor. Forskolin-stimulated cAMP values for each receptor and mock transfected cells (in nM) are given in brackets. Basal cAMP levels prior Forskolin-stimulation for mock-transfected cells were 0.88 \( \pm \) 0.57 nM. The number of assays is indicated in each column. All assays were performed in triplicates.

<table>
<thead>
<tr>
<th>transfected construct</th>
<th>agonist</th>
<th>cAMP inhibition % of FSK stimulation (n=10)</th>
<th>cAMP (nM) cotransfected G\textsubscript{α}_{si5} (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mock</td>
<td>FSK/basal</td>
<td>100 (7.78 ( \pm ) 1.29)</td>
<td>2.71 ( \pm ) 1.42</td>
</tr>
<tr>
<td></td>
<td>UDP</td>
<td>96.93 ( \pm ) 9.53</td>
<td>2.95 ( \pm ) 2.22</td>
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<tr>
<td></td>
<td>UDP-Glc</td>
<td>104.06 ( \pm ) 18.13</td>
<td>3.12 ( \pm ) 1.18</td>
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<tr>
<td></td>
<td>NPY</td>
<td>106.08 ( \pm ) 27.75</td>
<td>3.12 ( \pm ) 1.44</td>
</tr>
<tr>
<td></td>
<td>CCh</td>
<td>92.13 ( \pm ) 17.59</td>
<td>2.67 ( \pm ) 1.76</td>
</tr>
<tr>
<td>mouse P2Y\textsubscript{14}</td>
<td>FSK/basal</td>
<td>100 (8.96 ( \pm ) 1.92)</td>
<td>7.22 ( \pm ) 1.99</td>
</tr>
<tr>
<td></td>
<td>UDP</td>
<td>103.37 ( \pm ) 22.91</td>
<td>8.10 ( \pm ) 2.18</td>
</tr>
<tr>
<td></td>
<td>UDP-Glc</td>
<td>93.81 ( \pm ) 21.66</td>
<td>9.20 ( \pm ) 2.05</td>
</tr>
<tr>
<td>human P2Y\textsubscript{14}</td>
<td>FSK/basal</td>
<td>100 (8.73 ( \pm ) 3.12)</td>
<td>7.49 ( \pm ) 3.00</td>
</tr>
<tr>
<td></td>
<td>UDP</td>
<td>99.10 ( \pm ) 18.56</td>
<td>8.04 ( \pm ) 3.26</td>
</tr>
<tr>
<td></td>
<td>UDP-Glc</td>
<td>98.88 ( \pm ) 21.47</td>
<td>8.86 ( \pm ) 3.50</td>
</tr>
<tr>
<td>mouse Y2R</td>
<td>FSK/basal</td>
<td>100 (11.54 ( \pm ) 3.21)</td>
<td>4.18 ( \pm ) 0.98</td>
</tr>
<tr>
<td></td>
<td>NPY</td>
<td>64.49 ( \pm ) 15.09</td>
<td>12.10 ( \pm ) 3.24</td>
</tr>
<tr>
<td>human M2R</td>
<td>FSK/basal</td>
<td>100 (12.09 ( \pm ) 3.30)</td>
<td>4.93 ( \pm ) 0.56</td>
</tr>
<tr>
<td></td>
<td>CCh</td>
<td>80.00 ( \pm ) 21.28</td>
<td>11.34 ( \pm ) 2.96</td>
</tr>
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</table>
Next, COS-7 cells transfected with the human and mouse P2Y\textsubscript{14} were tested in classical cAMP inhibition assays where cAMP levels were increased by forskolin. In contrast to the experiments with chimeric G proteins no increased basal activity was seen in P2Y\textsubscript{14}-transfected cells (Table 8). Neither UDP nor UDP-glucose showed agonistic activity at P2Y\textsubscript{14} whereas stimulation of G\textsubscript{i}-coupled Y2 and muscarinic M2 receptors partially inhibited forskolin-induced cAMP formation (Table 8).

Finally, the mouse P2Y\textsubscript{14} was tested in a more sensitive and kinetic assay using dynamic mass redistribution (EPIC ®). Experiments showed no significant signal for UDP-glucose and UDP on mouse P2Y\textsubscript{14}-transfected compared to mock-transfected cells (Fig. 14B). However, the neuropeptide Y Y2 receptor (positive control) showed a robust transient after NPY stimulation (Fig. 14B).

**Figure 14 Testing UDP-glucose and UDP at P2Y\textsubscript{14} in yeast and mammalian cells.**

(A) Genetically engineered yeast cells (69) transformed with the indicated receptor constructs were analyzed without (basal) and with agonist (10 µM). Receptor activation was measured after 40 hours as growth of yeast cells in histidine-depleted medium by recording the optical density (OD) at 600 nm. Data (mean ± SD) from two independent experiments (in triplicate) are shown. (B) Transiently transfected HEK293 cells (P2Y\textsubscript{14}, neuropeptide Y receptor Y2) were seeded in 384-well plates the day prior DMR measurements (59). Cells were challenged with indicated concentrations of the corresponding agonist (UDP, UDP-glucose, NPY) and wavelength shift (in pm) was monitored. Each receptor stimulation curve was first corrected against own buffer control (basal) and second, against mock-transfected cells stimulated with the respective agonist. Data from three independent experiments (mean ± SEM) were summarized, each performed in quadruplicate.

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5 DISCUSSION

Although widely expressed, previous studies on P2Y₁₄ mainly focused on immune cells and its relevance in inflammatory responses (13,32,34,35,40-42). Initial evidence indicates a role of P2Y₁₄ in UDP-glucose-mediated chemotaxis of neutrophils and bone marrow cells, mast cell degranulation and proliferation of T cells (34,35,43,70). However, studies suggest that UDP-glucose can mediate cellular effects via P2Y₁₄-dependent and -independent pathways (45,71,72). To study the physiological function of P2Y₁₄ a KO mouse line was established by targeted deletion of this GPCR gene and replacement with a LacZ reporter gene.

5.1 P2Y₁₄ deficiency is compatible with life

The P2Y₁₄-deficient offspring was characterized in a primary screen searching for major anomalies in morphology and development. KO mice were vital and fertile. This is consistent with a very recent study investigating P2Y₁₄ relevance in embryonic lethality after radiation (44). Except for a significantly higher spleen weight (Table 1) and slightly increased blood pressure (Table 2) no obvious differences in genotype distribution, breeding, growth, gross morphology, histology, laboratory chemistry and behavior were detected on primary inspection in comparison to WT mice (Tables 3-5). Scrivens and Dickenson (35) focused in their study on the expression of P2Y₁₄ in spleen and characterized the involvement of this GPCR in T-cell function. Indeed, UDP-glucose was able to inhibit the proliferation of murine spleen-derived T-cells induced by IL-2 and anti-CD3 antibody treatment (35). In line with my data, Kook et al. (44) described higher spleen weights of P2Y₁₄-deficient offspring following prenatal radiation, suggesting a protective effect of P2Y₁₄ deficiency in preventing spleen cell damage. Controversially, in utero pretreatment of WT mice with the putative receptor agonist UDP-glucose mimicked the effect found in mice lacking P2Y₁₄ (44).

However, the rather mild phenotype of P2Y₁₄-deficient animals was observed under SPF conditions. It was shown that over 40% of all GPCR-deficient mouse models only display an obvious phenotype after challenging with a pathogen, drug or other challenging conditions (47). Therefore, tissue distribution analyses are often useful to obtain information about possible functions.
5.2 P2Y\textsubscript{14} is widely expressed in mouse tissues

Quantitative PCR analyses showed a ubiquitous expression of P2Y\textsubscript{14} in mouse tissues with high expression levels in pancreas, salivary glands, brain, lung and the GI tract and lowest expression in liver (Fig. 6A). Taking advantage of the introduced LacZ reporter construct the expression was tracked to a subpopulation of smooth muscle cells throughout the GI tract (Fig. 4). Several co-stainings with known smooth muscle cell markers revealed no overlap with the β-galactosidase-positive cells. To my best knowledge, there is no other smooth muscle subtype which may correspond to the cells marked by the P2Y\textsubscript{14}-reporter construct and may indicate a previously non-described smooth muscle cell subpopulation.

Reporter gene expression can, in principle, show adverse expression effects due to adaptation to the lack of the deleted gene. To address this issue P2Y\textsubscript{14} and LacZ reporter gene expression were studied in WT and KO mice. Quantitative PCR experiments showed that the mRNA expression levels and the tissue expression specificity of the reporter LacZ was similar to P2Y\textsubscript{14} (Fig. 6B). This equivalence of transcript levels almost excluded compensatory or reactive LacZ expression effects following P2Y\textsubscript{14} deficiency in KO tissues and showed that the reporter gene properly indicates P2Y\textsubscript{14} expression in tissues. Furthermore, it implicates that the reporter gene construct most probably does not influence the phenotypes that were studied in more detail.

The ubiquitous expression of P2Y\textsubscript{14} in mouse tissues is in accord with previous findings in human and rat tissues (32,39,40) but now adds that in many organs distinct smooth muscle cells are the cellular basis of broad expression levels previously found in qPCR and Western blot analyses.

5.3 P2Y\textsubscript{14} deficiency alters smooth muscle functions in GI tract and airways

To study the consequences of P2Y\textsubscript{14} deficiency in the different smooth muscle-containing organs several GI tract and bronchial tract functions were tested. A previous study in rat revealed high P2Y\textsubscript{14} mRNA levels and found UDP-glucose-dependent increase in the baseline muscle tension of the forestomach. However, comparison of WT and P2Y\textsubscript{14}-deficient mice revealed no differences in gastric emptying (45).

In studies with isolated intestine the contractility of several GI segments of KO mice was not different from those of WT mice following electric and carbachol stimulations. Moreover, UDP-glucose showed no effect on contraction of intestine preparations of WT mice.
However, observing the entire GI passage the lack of P2Y\textsubscript{14} reduced GI emptying (Fig. 7), suggesting a more distinct effect of P2Y\textsubscript{14} on smooth muscle function of the GI tract. Interestingly, P2Y\textsubscript{14}-expressing bronchioles were also affected by deficiency of this GPCR. Thus, P2Y\textsubscript{14}-KO mice displayed a higher dynamic compliance in response to inhaled methacholine (Fig. 8) during invasive plethysmography tests. Taken together, with the previously described role of P2Y\textsubscript{14} in immune and inflammatory responses this is an interesting finding as chronic pulmonary diseases like asthma or chronic obstructive pulmonary disease (COPD) show inflammatory and narrowing remodeling processes in the bronchioli and airway hyperresponsiveness (73,74). Indeed, it has been shown that sputum samples from patients with airway inflammation contain higher levels of UDP-glucose (75) and Müller et al. (76) described the involvement of P2Y\textsubscript{14} in release of chemokines like IL-8 from human airway epithelial cells. Interestingly, the involvement in asthma pathogenesis has been demonstrated for the ADP receptor P2Y\textsubscript{12}, another member of the P2Y\textsubscript{12}-like receptor group (77).

By analogy, differential expression of P2Y\textsubscript{14} was also found in intestinal biopsies of patients with chronic bowel diseases (78). This group of disorders comprises Crohn’s disease and ulcerative colitis and is characterized by intestinal inflammation (77). In fact, P2Y\textsubscript{14} was the only nucleotide receptor-related gene that displayed a positive expression correlation with grade of inflammation in ulcerative colitis (78). Further studies of P2Y\textsubscript{14} function in murine models of acute or chronic lung diseases and inflammatory bowel diseases are required to dissect its possible contribution to these disorders.

Furthermore, one should note, that P2Y\textsubscript{14} is expressed in venous blood vessels (Fig. 3) and P2Y\textsubscript{14} deficiency led to significant increases in blood pressure and heart rate (Table 2). There is some evidence that P2Y\textsubscript{14} may have vasocontractile function on arteries (79) but P2Y\textsubscript{14} expression in smooth muscle cells in arteries is controversially discussed (80,81). I found some ß-galactosidase positive arteries only in lung and CNS (circulus arteriosus Willisii) (data not shown). Future studies will address if there is a physiological link between P2Y\textsubscript{14} expression in the vascular system and hemodynamic functions.

In sum, these studies show that P2Y\textsubscript{14} is highly expressed in a distinct subset of smooth muscle cells. P2Y\textsubscript{14} expressed on those cells modulate contractile function of the GI and the bronchial tracts.
5.4 P2Y_{14} deficiency results in reduced glucose tolerance

The most obvious phenotype was the reduced oral glucose tolerance (Fig. 9A/C) in P2Y_{14}-deficient mice. Altered glucose resorption, reduced insulin sensitivity and decreased pancreatic insulin secretion can contribute to an impaired oral glucose tolerance. Resorptioanal problems in the GI tract could not account for the reduced oral glucose tolerance since P2Y_{14}-KO mice also showed significantly higher blood glucose levels following intraperitoneal glucose injection (Fig. 9B/D). The observed reduced glucose tolerance in P2Y_{14}-deficient mice more likely results from the lower pancreatic insulin secretion after glucose application (Fig. 10C/D). In a recent paper, Xu et al. (82) showed improved glucose tolerance in P2Y_{14}-deficient mice fed with a high-fat diet due to an elevated insulin sensitivity e.g. of the liver. This is in contrast to my data where insulin sensitivity of KO and WT mice was not different (Fig. 10A/B). It was argued that reduced macrophage infiltration and hepatic inflammation account for improved hepatic insulin sensitivity (82). As in my study (Fig. 10C/D), Xu et al. (82) found reduced serum insulin levels after glucose administration in KO mice. This important finding remained unappreciated in their study and other pathophysiological mechanisms were not considered. In a more reasonable scenario, lower insulin levels result in a reduced hepatic steatosis, as found in (82) under high-fat diet. In fact, the differential expression of genes involved in lipid and glycogen metabolism in liver, as found in (82), can be interpreted as a consequence of lower insulin levels. It is well known that insulin stimulates the expression of lipogenic enzymes such as fatty-acid synthase and acetyl-CoA carboxylase and inhibits the transcription of gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (83). The hepatic inflammation is most likely secondary to the alimentary hepatic steatosis found in Xu et al (82). Since the western type diet I used in my experiments does not significantly differ from the high-fat diet (see 3.5) Xu et al. fed the mice (26.3% carbohydrates, 34.9% fat, 26.2% protein) I further focused on the reason for the lower blood insulin levels (Fig. 10 C/D) which were seen already in animals fed with standard chow.

5.5 Reduced insulin release from P2Y_{14}-deficient islets

Since morphological changes and altered number of pancreatic islets did not account for the reduced serum insulin levels in P2Y_{14}-deficient mice, differences at the molecular level were studied in detail using a transcriptome-wide approach.
In line with β-galacosidase staining of pancreatic tissue (Fig. 5D), the RNA-sequencing data (Table 6), also qPCR analysis (ΔCt = 7.64 ± 0.55), and other studies (46) clearly demonstrate P2Y\textsubscript{14} expression in pancreatic islets. Transcriptome data showed significant changes in relevant components of glucose sensing, Ca\textsuperscript{2+}-triggered and GPCR-modulated insulin release pathways (Fig. 12) in islets of P2Y\textsubscript{14}-deficient mice which suggested changes in islet function of KO animals and indeed, \textit{in vitro} experiments revealed a negative impact of P2Y\textsubscript{14} deficiency on glucose-dependent insulin release from isolated pancreatic islets (Fig. 13) and confirmed my \textit{in vivo} findings.

Since P2Y\textsubscript{14} couples to G\textsubscript{i/o} proteins, inhibits adenylyl cyclases and mobilizes intracellular Ca\textsuperscript{2+} (41,43) most probably via release of G\textsubscript{βγ} dimers from G\textsubscript{i} proteins (84), one would expect that loss of this gene should rather improve glucose-mediated insulin release from β-cells. However, UDP-glucose alone was neither able to significantly release insulin nor significantly reduce glucose-induced insulin release from isolated islets (see Fig. 13B-D). Therefore, I re-evaluated the agonistic properties of UDP- and UDP-glucose at the mouse and human P2Y\textsubscript{14} with different methods \textit{in vitro}. Techniques using chimeric G proteins in yeast and mammalian heterologous expression systems showed high basal activity of P2Y\textsubscript{14} and some minor UDP- and UDP-glucose-mediated responses (Fig. 14A, Table 8). However, UDP and UDP-glucose did not significantly activate P2Y\textsubscript{14} in classic cAMP inhibition assay (Table 8) and label-free DMR (Fig. 14B), whereas the G\textsubscript{i}-coupled neuropeptide Y receptor Y2 showed robust agonist-mediated signals in all tests. At the current stage, UDP and UDP-glucose show weak agonistic activity at P2Y\textsubscript{14} in functional assays using chimeric G proteins (30). Label-free and classic second messenger assays as well as my \textit{in vivo} data with knock-out mice do not provide evidence for physiological activities of UDP and UDP-glucose at P2Y\textsubscript{14}. Further studies using clear-cut controls (e.g. gene-deficient animals) are required to finally prove or disprove UDP-Glc and UDP as physiological agonists for P2Y\textsubscript{14}.

The cell type within Langerhans islets, where a specific GPCR is expressed, determines its function on insulin exocytosis. Currently, the cell type(s) of pancreatic islets expressing P2Y\textsubscript{14} is unknown. Further, the effect of a given GPCR on insulin release apparently not only depends on its G protein-coupling mode. Thus, G\textsubscript{q} protein-coupled receptors such as the GnRH receptor inhibits insulin release whereas the exclusively G\textsubscript{i} protein-coupled cannabinoid receptor type 1 and the melanin-concentrating hormone receptor stimulate insulin release from islets (46). The most abundant somatostatin receptor SSTR3 (see Table 6), which is G\textsubscript{i/o} protein-coupled, has no significant effect on insulin release (46). As for several GPCR
expressed in pancreatic islets (46) the specific signaling pathway how P2Y\textsubscript{14} modulates gene expression and functions of islets needs to be studied further.

5.6 Conclusion

Mice with P2Y\textsubscript{14} deficiency present a rather mild phenotype under SPF conditions. However, my studies revealed involvement of P2Y\textsubscript{14} in GI tract emptying and glucose homeostasis regulation. The lack of P2Y\textsubscript{14} function reduced intestine passage and glucose-stimulated insulin release from pancreatic islets \textit{in vitro} and \textit{in vivo}. It is of interest that several GPCR, e.g. GLP1R, GPR39, M3 muscarinic receptor and galanin receptor, modulate both, GI motility and glucose-induced insulin secretion (85-88). For P2Y\textsubscript{14} it is currently unclear whether this GPCR realizes its effect by acute activation of its signaling cascade or during islet ontogenesis. The gross transcriptome changes found in P2Y\textsubscript{14}-deficient islets may also suggest a more complex alteration of the expression profile indicating a role of P2Y\textsubscript{14} in subdifferentiation of islets cells. Indeed, some genes which are involved in differentiation and maturation of pancreatic islet cell types (e.g. MAFA, MAFB, REG2) (89-91) are differentially expressed in the islets isolated from P2Y\textsubscript{14}-deficient mice (Fig. 12). Although there were no differences in gross islet morphology and islet size distribution (Fig. 11), there is evidence that e.g. pancreatic β-cells are not homogenous but rather present functionally different subpopulations with distinguishable functionality (92,93). Thus, P2Y\textsubscript{14}-mediated signaling may be one signal that modulates distinct functionalities of islet cells. Since Langerhans islets derived from pancreatic ductal cells during ontogenesis and P2Y\textsubscript{14} expression was morphologically also found in the exocrine pancreas (Fig. 5A-C), more detailed studies should focus on the relevance of P2Y\textsubscript{14} in pancreas development. Further studies with conditional β-cell-specific KO mice can help to differentiate between acute and adaptive effects of P2Y\textsubscript{14} deficiency. Moreover, P2Y\textsubscript{14} gene variants may contribute to a prediabetic state that enhances the risk of metabolic disease in humans. This obviously raises the questions whether there are polymorphisms of the P2Y\textsubscript{14} gene within the human population that define this risk and whether signatures of recent selection can be found at the locus. Nevertheless, findings in this study identify P2Y\textsubscript{14} as a novel modulator of insulin secretion.
6 SUMMARY

Dissertation zur Erlangung des akademischen Grades

Dr. med.

Titel: The physiological relevance of the G protein-coupled receptor P2Y14

eingereicht von: Jaroslawna Meister

angefertigt am: Institut für Biochemie
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eingereicht im: 07/2014

G protein-coupled receptors (GPCR) play a significant role in regulating virtual all physiological functions and more than one third of currently clinically used drugs target GPCR. However, there are approximately one hundred so-called orphan GPCR to whom neither the physiological function nor the endogenous ligand has been assigned yet. Therefore, the identification of their role in organism could display new targets for therapeutic applications.

The UDP-glucose receptor P2Y14 is a GPCR that belongs to the family of the P2Y12-like receptors due to phylogenetic relation. This group comprises among other receptors the clopidogrel-sensitive ADP receptor P2Y12 whose pivotal role in platelet aggregation and immune function has been well characterized in several studies. However, only little is known about the physiological function of other GPCR of the P2Y12-like receptor group. Previous studies on P2Y14 function focused on its involvement in immune and inflammatory responses based on the high abundance of this GPCR in the immune system. Because high expression was found in the gastrointestinal (GI) system, it seemed reasonable to investigate a possible role of P2Y14 in the regulation of nutrient uptake and energy metabolism. Taking advantage
of a gene-deficient (KO) mouse strain this study aimed at the characterization of the biological consequences of UDP-glucose receptor deficiency.

The study revealed the following main results:

1. The generated P2Y\textsubscript{14}-KO mouse strain showed no obvious phenotypes regarding genotype distribution, breeding, growth, development and morphology. The P2Y\textsubscript{14}-deficient offspring was vital and fertile and displayed no gross differences in laboratory screens and behavioral tests except for a higher spleen weight and increased blood pressure and heart rate under specific pathogen free (SPF) conditions.

2. P2Y\textsubscript{14} is widely expressed in mouse tissues showing high expression levels in pancreas, salivary glands, brain, lung, and parts of the GI tract and lowest expression in liver. Using a LacZ reporter gene the expression of the P2Y\textsubscript{14} was tracked to distinct smooth muscle cells within the GI tract. This smooth muscle cell population is different to any other described subpopulations (e.g. Cajal cells).

3. P2Y\textsubscript{14} deficiency in mice results in altered smooth muscle function in the GI and bronchial tracts. P2Y\textsubscript{14}-KO mice show a prolonged GI passage in GI transit experiments. In an invasive plethysmography test mice deficient for P2Y\textsubscript{14} showed enhanced lung compliance following increasing doses of inhaled methacholine. These results indicate a role of P2Y\textsubscript{14} in modulating the contractile function of smooth muscle cells expressing this GPCR.

4. Metabolic studies revealed a reduced oral and intraperitoneal glucose tolerance in P2Y\textsubscript{14}-KO mice kept under standard and western type diet. By performing insulin tolerance and insulin secretion tests this phenotype was pinpointed to lower serum insulin levels following glucose application in mice lacking P2Y\textsubscript{14}.

5. Morphometric analyses of pancreatic tissue displayed no differences in number and size of the islets of P2Y\textsubscript{14}-KO mice in comparison to WT mice, suggesting no gross morphological abnormalities in islet development.

6. To assess possible differences in islets of P2Y\textsubscript{14}-KO mice at the molecular level RNA expression was analyzed in a transcriptome-wide approach using RNA-sequencing. 815 genes were found differentially expressed between the islets of KO and WT mice. Transcriptome data show significant alterations in expression of components relevant for glucose sensing, Ca\textsuperscript{2+}-triggered and GPCR-modulated
insulin release pathways in islets of P2Y$_{14}$-deficient mice suggesting changes in islet function of KO animals.

7. *In vitro* insulin secretion experiments demonstrated a reduced insulin release from isolated pancreatic islets of P2Y$_{14}$-deficient mice despite similar amount of produced insulin in islets isolated from both WT and KO mice.

In conclusion, mice with P2Y$_{14}$ deficiency present a rather mild phenotype with higher spleen weight and delayed GI tract emptying under SPF conditions. However, more detailed analysis of metabolic parameters revealed a significantly reduced glucose tolerance and insulin secretion in KO mice. In addition to immune function these metabolic data indicate a role of P2Y$_{14}$ in the regulation of energy homeostasis via modulation of the insulin release.
7 REFERENCES


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8 SUPPLEMENTAL MATERIAL

(FIRST AUTHOR PUBLICATION)
Metabolism:
The G Protein-coupled Receptor P2Y14 Influences Insulin Release and Smooth Muscle Function in Mice

Jaroslawa Meister, Diana Le Duc, Albert Ricken, Ralph Burkhardt, Joachim Thiery, Helga Pfannkuche, Tobias Polte, Johannes Grosse, Torsten Schöneberg and Angela Schulz

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The G Protein-coupled Receptor P2Y14 Influences Insulin Release and Smooth Muscle Function in Mice

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Keywords: G protein-coupled receptors, gene knockout, glucose metabolism, pancreatic islets, signal transduction

Background: The relevance of the widely expressed GPCR P2Y14 is only partially understood.

Results: Analysis of P2Y14 KO mice revealed decreased gastrointestinal emptying, reduced glucose tolerance and insulin release.

Conclusion: P2Y14 function is required for proper intestine emptying and adequate glucose response.

Significance: P2Y14 plays a role in smooth muscle function and maintaining energy homeostasis by influencing insulin release.

ABSTRACT

UDP-sugars were identified as extracellular signaling molecules, assigning a new function to these compounds in addition to their well defined role in intracellular substrate metabolism and storage. Previously regarded as an orphan receptor, the G protein-coupled receptor (GPCR) P2Y14 (GPR105) was found to bind extracellular UDP and UDP-sugars. Little is known about the physiological functions of this GPCR. To study its physiological role we used a gene-deficient (KO) mouse strain expressing the bacterial LacZ reporter gene to monitor the physiological expression pattern of P2Y14. We found that P2Y14 is mainly expressed in pancreas and salivary glands and in subpopulations of smooth muscle cells of the gastrointestinal tract, blood vessels, lung and uterus. Among other phenotypical differences KO mice showed a significantly impaired glucose tolerance following oral and intraperitoneal glucose application. An unchanged insulin tolerance suggested altered pancreatic islet function. Transcriptome analysis of pancreatic islets showed that P2Y14 deficiency significantly changed expression of components involved in insulin secretion. Insulin secretion tests revealed a reduced insulin release from P2Y14-deficient islets highlighting P2Y14 as a new modulator of proper insulin secretion.

Although the physiological function is known for many members of the family of GPCR, functional ligands have not been identified yet for more than one hundred so-called orphan receptors. In the past years, academic research groups and pharmaceutical companies engaged in large-scale deorphanization efforts for these receptors employing ligand screening studies and analyses of receptor-deficient mouse models (1). One of these studies revealed P2Y14 (formerly named GPR105)
as a receptor that is activated by UDP-glucose and closely related sugars (2,3). This was an interesting finding as UDP-glucose is a pivotal metabolite in intracellular glucose storage pathways including glycogen biosynthesis. In more recent studies, UDP alone was described as ligand for P2Y14 (4,5), however, another group tested the agonistic activity of UDP but found no activation of P2Y14 (2). P2Y14 belongs to the P2Y12–like receptor group within the family of rhodopsin-like receptors. The P2Y12–like receptor group comprises the ADP receptors P2Y12 and P2Y13, P2Y14 and the receptors GPR34, GPR82, GPR87, and GPR171 (6). P2Y14 mRNA expression analysis showed a widespread expression pattern including high expression levels in placenta, adipose tissue, stomach and intestine and moderate levels in spleen, lung, heart and different brain regions (3,7). P2Y14 was also detected in immune cells like B- and T-lymphocytes, neutrophils, dendritic cells, astrocytes, and microglia (7-11) suggesting a physiological function in immune response. Indeed, expression of P2Y14 was upregulated in response to inflammatory injury (12) and similar results were shown by in vivo treatment with lipopolysaccharide for rat brain and spleen (7,13). However, as the strongest expression was found in the gastrointestinal (GI) system, we explored a possible role of P2Y14 in the regulation of nutrient uptake and energy metabolism.

EXPERIMENTAL PROCEDURES

Cell culture, transfection and functional assays – For yeast experiments, the haploid Saccharomyces cerevisiae yeast strain MPY578t5 (provided by Dr. Mark Pausch) was used for the expression of receptor constructs. Cells were transfected with plasmid DNA using electroporation as described (14). For expression in mammalian cells, COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified 5% CO2 incubator. Transient transfection experiments of COS-7 cells with receptor constructs for cAMP inhibition measurements and for direct cAMP measurements (co-transfected with the chimeric G protein Gαs5) were essentially performed as described previously (15). For measurements of dynamic mass redistribution (DMR) (16) HEK293 cells were grown in DMEM/F12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO2 incubator and transiently transfected with the receptor constructs and split onto fibronectin-coated 384-well plates one day prior the assay. DMR measurements were performed with the Epic system (Corning, NY, US).

For all transfection experiments the mouse and human P2Y14 coding sequence attached to an N-terminal Hemagglutinin epitope and a C-terminal FLAG epitope was cloned into the mammalian expression vector pcD-ps. Additionally, for yeast experiments the mouse receptor was introduced into the yeast vector pCD-Ps. All control receptor plasmids were cloned with the described strategy.

Generation of P2Y14-deficient mice - P2Y14-KO mice were generated at TAKEDA Ltd. (Cambridge, United Kingdom) by targeted disruption of the mouse P2Y14 locus and ES-cell blastocyst injection. Thus, most part of the coding region of P2Y14 was exchanged by a β-galactosidase cassette harbouring an internal ribosome entry site (IRES) followed by a loxP-flanked neomycin cassette (Fig. 1). After removal of the neomycin cassette, the resulting KO animals were bred with 129S6 animals and all studies were done on a mixed C57BL/6 x 129S6 background with predominance of 129S6. For experiments WT and KO littermates from intercrossed WT and KO parents were used. Genotyping of mice was carried out by PCR with the following primers: β-gal sense (5’-AGAAGGCACATGGCTGAATATCGA-3’), forward (5’-AGCTGCCGGACGAAGGAGACCCTGCTC-3’) and reverse (5’-GGTTTTGAAACCTCTAGGTCATTCTG-3’) in two separate PCR reactions (Fig. 1): forward/reverse to amplify WT allele (180 bp) and β-gal sense/reverse to amplify KO allele (400 bp). The PCR conditions were 95°C for 3 minutes followed by 35 cycles with 45 sec 95°C, 60°C 30 sec and 72°C for 1 min and a final amplification step of 72°C for 10 min.

All mice were maintained in a specific pathogen-free barrier facility on a 12-h light/12-h dark cycle with ad libitum access to water and food. Experiments were performed according to the accepted standards of animal care and were
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Quantitative expression analysis - Several tissues were removed from 3 WT and 3 KO mice and RNA was isolated using TRIZOL (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer’s instructions. RNA quantity was measured with a spectrophotometer (Nanodrop ND 1000, NanoDrop products, Wilmington, USA) and RNA quality was controlled by gel electrophoresis. For quantitative real time PCR (qPCR) 1 µg of total RNA was reversely transcribed (Superscript II RT, Invitrogen, Karlsruhe, Germany) using oligo-dT primers. qPCR was performed by Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to manufacturer’s instructions. Primers were designed for the intron-flanking exons 1 and 2 sequences (Fig. 1) to exclude genomic contaminations (sense: 5’-GAAGCCAGACGTGAAGGAGTT-3’; antisense: 5’-CAGGAATCTCAAAGGCAAGCT-3’) resulting in a 156-bp product. qPCR was performed with the MX 3000P instrument (Agilent Technologies GmbH, Boeblingen, Germany) using the following protocol: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 15 s at 95 °C and 30 s at 60 °C. A product dissociation curve was recorded to verify the presence of a single amplicon. Threshold cycle (Ct) values were determined during the exponential increase of the product in the PCR. After normalization to the house keeping gene β2-microglobulin calculated ∆Ct values were used to determine the relative expression of P2Y14.

LacZ reporter gene assay - Tissue samples of several organs were prepared from WT and KO mice, covered in Tissue-Tek® (Sakura, Torrance, CA, USA) and snap frozen in liquid nitrogen. The organs were cryosectioned (10 µm) and fixed in acetone-methanol (1:1) solution. Sections were incubated with X-Gal staining solution at 30 °C overnight to detect the expression of the LacZ reporter gene via β-galactosidase activity.

Laboratory chemistry/histology/behavioral tests – Serum levels of electrolytes, metabolites, enzymes and hormones were analyzed in 3-month-old WT and KO mice according to the guidelines of the German Society of Clinical Chemistry and Laboratory Medicine, using a Hitachi PPE-Modular analyzer (Roche Diagnostics, Mannheim, Germany). Histological slices (5 µm) were prepared from organs being fixed in 4% formaldehyde solution and embedded in paraffin wax. Slices were stained with H&E. Blood cell counting from EDTA blood samples was performed automatically (ScilVet abc; Scil corporation, Viernheim, Germany). Open field and hot plate tests were performed as reported previously (17) using automated measuring technology (TSE Systems, Bad Homburg, Germany; Hot Plate 602001, TSE)).

Gastrointestinal transit and contractility studies - A gastrointestinal emptying test was performed by oral application of 200 µl dye-labeled non-absorbable liquid dextran blue (20 mg/ml) to mice. Faeces were collected hourly for 8 hours, vortexed with 300 µl phosphate buffered saline (PBS) and centrifuged at 13,000 rpm for 10 min. The amount of hourly excreted dextran blue was measured photometrical at 620 nm in supernatants. Mice that showed no excretion for at least 2 hours were excluded from the experiment. For contractility studies mice were sacrificed by cervical dislocation. The intestine from mice was harvested and placed in ice-cold Krebs-Ringer buffer (KRB). One cm long equal segments of intestine were placed in 50-ml organ baths (37 °C) containing KRB solution and continuously gassed with 95% O2 and 5% CO2. To record the contractile activity, each intestine segment was connected to an isometric force transducer (MLT0201 Force Transducer; ADInstruments GmbH, Spechbach, Germany). The basal tension was set to 10 mN. Several parameters like mean muscle tension, average tension maximum and minimum, average amplitude, rate of contractions and the area under the curve and the effect of 100 µM UDP-glucose and 100 µM carbachol (positive control) were analyzed using Lab Chart software (ADInstruments). Data were normalized to recorded spontaneous activity and expressed in percentage.

Hemodynamic parameters - Blood pressure and pulse were determined by non-invasive tail-cuff method using the BP-2000 Blood Pressure Analysis System™ (Visitech Systems, Apex, NC, USA). Following manufacturer’s instructions, mice were trained to the procedure each day for at least 5 days prior to the actual experiment. After the training period 10 measurements per day of each mouse on 5 consecutive days were performed. Systolic blood pressure, diastolic blood pressure, mean arterial pressure, and pulse were calculated from daily experiments for each mouse.
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Airway responsiveness - Lung resistance and dynamic compliance were measured by invasive plethysmography (emka TECHNOLOGIES, Paris, France) in response to inhaled UDP-glucose and methacholine (Sigma-Aldrich). Female mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine; Bayer Leverkusen, Germany), intubated and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline lung resistance, dynamic compliance, and responses to aerosolized saline (0.9 % NaCl) were measured first, followed by responses to 100 µM aerosolized UDP-glucose and increasing doses (10 to 80 mg/ml) of aerosolized methacholine (18).

Metabolic studies - 1-month-old mice were kept on standard (Ssniff M-Z: 5% sugar, 4.5% raw fat, 34% starch, 22% raw protein) or western (Ssniff EF R/M TD88137: 32.8% sugar, 21.2% raw fat, 14.5% starch, 17.1% raw protein) type diet (Ssniff GmbH, Soest, Germany) for 3 months. During this period body-weight development was monitored weekly. For glucose tolerance tests mice on a standard or a western type diet had been fasted overnight (15 h) and blood glucose levels were measured before (0 min), 20, 40, 60 and 120 min after oral application (80 mg) or intraperitoneal injection (2 mg/g body weight) of glucose. For insulin tolerance tests blood glucose levels were measured before (0 min) and 15, 30 and 60 min after intraperitoneal injection of human insulin (0.75 U/kg body weight). Blood glucose levels were determined by using an automated blood glucose meter (FreestyleLite, Abbott Diabetes Care Ltd., Oxon, UK). For insulin secretion tests mice fasted overnight were injected intraperitoneally with glucose (2 mg/g body weight) and blood samples were taken before (0 min), 20, 40 and 60 min after injection. Serum insulin concentrations were measured using an Ultra Sensitive Mouse Insulin Elisa Kit (Crystal Chem, Inc., Downers Grove, IL, USA). All metabolic tests were performed with the same set of animals (one-week interval between the tests).

Morphometric analysis - Pancreata from 5 WT and 5 KO mice fed with western type diet were fixed in 4 % formaldehyde and embedded in paraffin wax. Two longitudinal sections (4 µm) were generated every 100 µm of tissue. Each time, the first section was stained with H&E, the second immunostained for insulin with the avidin-biotin complex technique using anti-insulin monoclonal rabbit antibody (100 µg/ml, Cell Signalling, Millipore, Germany) and goat anti-rabbit secondary antibody (7.5 mg/ml, Vector Laboraties, Biozol Diagnostica, Germany). Both sections were photographed with a Zeiss Axioimager Z1 microscope (Carl Zeiss Jena GmbH, Jena, Germany). Ten H&E stained sections per mouse were analyzed for number and area size of pancreatic islets using NIH ImageJ software. For determination of b-cell area per islet the insulin-positive area of at least 50 islets per mouse was calculated using NIH ImageJ software and related to the total islet area of the investigated slides.

Isolation of pancreatic islets and insulin secretion experiments - Pancreatic islets were isolated as previously described (19-21). Briefly, mice were sacrificed by cervical dislocation and the pancreas was inflated by intraductal injection of ice-cold DMEM media containing 0.2 mg/ml (1.08 Wünsch Units) Liberase TL Research Grade (Roche Diagnostics GmbH, Mannheim, Germany). Distended pancreas was digested by shaking in a 37 °C water bath for 17 minutes. Isolated islets were washed and picked under a stereomicroscope and incubated overnight at 37°C and 5% CO2 in RPMI-1640 media containing fetal bovine serum for insulin secretion studies. For dynamic insulin secretion studies groups of 50 pancreatic islets were placed in a perfusion chamber and continuously perfused with KRB for 30 min (flow: 1 ml/min). After this equilibration phase islets were perfused for 30 min with low glucose (2.8 mM) to determine the basal insulin release and then challenged for 30 min with 16.7 mM glucose or 16.7 mM glucose plus 100 µM UDP-glucose. Samples of perfusate were collected every minute and insulin concentration was determined at indicated time points by Insulin AlphaLISA Kit (Perkin Elmer, Rodgau, Germany). For static incubation studies groups of 5 islets were placed in a 96-well plate. After a 30-minute equilibration period in KRB solution containing 2.8 mM glucose, pancreatic islets were stimulated with KRB solution containing either 2.8 mM or 16.7 mM glucose or 16.7 mM glucose plus 100 µM UDP-glucose. The supernatants were then collected for measurement of secreted insulin and islets were lysed in acid-ethanol to extract the residual islet insulin (19,20). Insulin concentrations were determined by Insulin AlphaLISA Kit (Perkin Elmer).
RNA-sequencing of pancreatic islets - Total RNA from isolated pancreatic islets (10 male mice per genotype) was extracted by using TRIZOL (Sigma-Aldrich) according to the manufacturer’s instructions and RNA quantity was measured using a spectrophotometer (Nanodrop ND 1000). The RNA quality of all samples was analyzed on the Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip. cDNA libraries were generated using TruSeq RNA Sample Preparation Kits v2 (Illumina, San Diego, CA, USA) according to the manufacturer’s protocol. Indexed islet libraries of good quality were pooled and used for sequencing on two flow cell lanes on an Illumina HiScanSQ System. To confirm base calling correctness the raw data passed a quality check using FastQC software. It revealed low PHRED-scale scores at the 3’ end of the 101 bp long reverse reads. These reads were trimmed to a size of 80 bp. Then, reads were assigned to the individual animals using the ligated adapter indices. Further, the samples proceeded filtering to trim the adapter sequences and remove low-quality reads which were shorter than 60 bp or contained more than five bases with a quality score below 15 (PHRED-scale). The paired-end reads were mapped to the reference mouse genome (July 2007 NCBI37/mm9) with Ensembl v66 annotations using Tophat 1.3.3. (22), which aligns reads using Bowtie (version 0.9.9). Mitochondrial reads and reads which did not map uniquely to a genome position were excluded. FPKM (fragments per kilobase of transcript per million mapped reads) values were calculated using Cufflinks 1.3.0 (22,23). Analysis of differential expression was performed using DESeq software package (24). Only genes that were expressed at least in 10 animals were considered for analysis. Functional enrichment analysis of differentially expressed genes (P < 0.05) was determined using ToppGene Suite (25).

RESULTS AND DISCUSSION

Although widely expressed, previous studies on P2Y14 mainly focused on immune cells and its relevance in inflammatory responses (7-12,26). Initial evidence indicates a role of P2Y14 in UDP-glucose-mediated chemotaxis of neutrophils and bone marrow cells, mast cell degranulation and proliferation of T cells (9,10,27,28). However, studies suggest that UDP-glucose can mediate cellular effects via P2Y14-dependent and -independent pathways (29-31). To identify phenotypes related to the lack of P2Y14 function, this GPCR gene was removed by targeted deletion and replacement with a LacZ reporter gene. The P2Y14-deficient offspring was vital and fertile. This is consistent with a very recent study investigating P2Y14 relevance in embryonic lethality after radiation (32). Except for a significantly higher spleen weight (suppl. Table S1) and slightly increased blood pressure (suppl. Table S1) no obvious differences in genotype distribution, breeding, growth, gross morphology, histology, laboratory chemistry and behavior were detected on primary inspection compared to WT mice (suppl. Table S1).

P2Y14 is widely expressed in mouse tissues – We next took advantage of the introduced LacZ reporter construct and monitored β-galactosidase activity in P2Y14-deficient mouse tissues. Whereas WT control tissues showed no specific staining, β-galactosidase-positive staining was found in uterus, bronchioles, blood vessels, pancreas, salivary glands (Figs. 2A and 3), and immune cells. Interestingly, staining in the GI tract was positive only in a subpopulation of smooth muscle cells (Fig. 2B/C). In all parts of the GI tract smooth muscle cells of the thin layer of the muscularis mucosae were β-galactosidase positive (Fig. 2B/C). From the ileum to the rectum a subpopulation of smooth muscle cells within the circular smooth muscle layer showed positive staining (Fig. 2B/C). In the rectum also the longitudinal smooth muscle layer displayed intensive β-galactosidase activity (Fig. 2B/C). Co-staining of c-kit, which is a marker of Cajal cells and mast cells in the GI tract (33,34), revealed no obvious overlap with the β-galactosidase-positive structures (data not shown). In contrast the β-galactosidase staining was partly reminiscent of the reported divergent reactivity of intestinal smooth muscle cells with characteristic smooth muscle cell markers (i.e. alpha-smooth muscle actin (ASMA), gamma-smooth muscle actin (GSMA)) (35). To our best knowledge, there is no other smooth muscle subtype which may correspond to the cells marked by the P2Y14-reporter construct.

Further assessment of the LacZ expression in pancreatic tissue showed β-galactosidase-positive staining in exocrine and endocrine glandular structures (Fig. 3). Interestingly, cells next to the pancreatic acini (Fig. 3A-C) and myoepithelial
cells of the salivary glands (Fig. 2A) were stained. Investigations addressing the specific functions of the myoepithelial cells are ongoing.

One should keep in mind that P2Y14-promoter-driven β-galactosidase-expression was monitored in KO mice and may reflect transcript over- or even non-physiological expression due to P2Y14 deficiency. To address this issue, we measured expression of the 5’UTR region of WT and KO P2Y14 gene transcripts, which were structurally similar in both WT and KO animals, by qPCR in a number of tissues (Fig. 4). As presented in Fig. 4B, ΔCt values of transcript expression showed a high correlation between WT and KO in all tissues (r² = 0.9058). This almost excluded compensatory or reactive LacZ expression effects following P2Y14 deficiency in KO tissues. Both data sets revealed that P2Y14 is widely expressed in mouse tissues with high expression levels in pancreas, salivary glands, brain, lung and parts of the gastrointestinal tract and lowest expression in liver (Fig. 4). This is in accord with previous findings in human and rat tissues (3,7,8) but now adds that distinct smooth muscle cells and immune cells are the cellular basis of broad expression levels previously found in qPCR and Western blot analyses.

P2Y14 deficiency alters smooth muscle functions in GI tract and airways – To study the consequences of P2Y14 deficiency in the different smooth muscle containing organs we tested several GI tract and bronchial tract functions. A previous study in rat revealed high P2Y14 mRNA levels and found UDP-glucose-dependent increase in the baseline muscle tension of the forestomach. However, comparison of WT and P2Y14-deficient mice revealed no differences in gastric emptying (30). Because we found no effect of UDP-glucose (up to 1 mM) on basal and carbachol-stimulated and electric-stimulated contraction of isolated small intestine and colon (data not shown), we speculated that the contribution of P2Y14 is more distinct and may present its significance only when the entire GI passage is observed. Thus, a dye-labeled dextran was applied to mice orally and the excretion in faeces was measured hourly. In comparison to WT animals the expulsion of dextran blue was significantly delayed in KO mice (Fig. 5). Interestingly, more KO animals (5 KO vs. 1 WT) had to be excluded from analysis according to our exclusion criterion (see Experimental Procedures).

P2Y14-reporter gene expression was also found in smooth muscle cells of bronchioles (Fig. 2A). To dissect its function in respiratory tract invasive plethysmography tests were performed. Although UDP-glucose had no effect on respiratory functions tested, KO mice showed significantly higher dynamic compliance in response to increasing inhaled doses of methacholine (Fig. 6).

One should note the expression of P2Y14 in mainly venous blood vessels (Fig. 2A) and significant increases in blood pressure and heart rate of P2Y14-deficient mice (suppl. Table S1). There is some evidence that P2Y14 may have vasocontractile function on arteries (36) but P2Y14 expression in smooth muscle cells in arteries is controversially discussed (37,38). We found some β-galactosidase positive arteries only in lung and CNS (circulus arteriosus Willisii) (data not shown). Future studies will address if there is a physiological link between P2Y14 expression in the vascular system and hemodynamic functions.

In sum, we show that P2Y14 is highly expressed in a distinct subset of smooth muscle cells. P2Y14 expressed on those cells modulate contractile function of the GI and the bronchial tracts.

P2Y14 deficiency results in reduced glucose tolerance – Our phenotype screen also included basic metabolic tests. Following oral glucose application KO mice showed significantly higher transient blood glucose levels (Fig. 7A). To differentiate whether the enteral glucose resorption was increased in KO mice due to the prolonged gastrointestinal emptying (see above), intraperitoneal glucose tolerance tests were performed. Blood glucose levels were also increased in KO mice following intraperitoneal glucose injection (Fig. 7B) excluding differences in enteral glucose resorption. To study whether high caloric nutrition can aggravate the phenotype found in KO mice, 4-week-old mice were kept on a western type diet for 3 months. WT and KO mice gained significantly body weight under the western type diet. But whereas there were no alterations in body weight development between WT and KO mice kept on standard diet, KO mice fed the western diet gained significantly less weight (KO: 178 ± 15% vs. WT: 196 ± 23%, P < 0.05). However, detailed analysis of serum cholesterol and TAG levels in all lipoprotein fractions revealed no differences between WT and KO mice fed a western type diet (suppl. Table S1).
Reduced insulin release in \( \text{P2Y}_{14}^-\) mice

Interestingly, glucose tolerances following oral and intraperitoneal glucose application were significantly reduced in KO mice kept under western type diet (Fig. 7C/D).

Next, we performed insulin tolerance tests to evaluate whether the higher glucose levels observed in KO mice in the oGTT were because of altered insulin sensitivity. There were no differences in the decline of blood glucose levels following intraperitoneal insulin injection between WT and KO mice (Fig. 8A). Similar results were found for mice kept on western type diet (Fig. 8B) although both genotypes showed lower insulin sensitivity as a result of higher body weight due to high caloric nutrition. As insulin sensitivity did not account for altered oGTT in KO mice we analyzed insulin levels in WT and KO mice during intraperitoneal glucose tolerance tests. On chow diet, KO mice (16 weeks old) tended to have lower serum insulin levels after intraperitoneal glucose application (Fig. 8C). Interestingly, after 12 weeks of western type diet glucose-stimulated insulin secretion of KO mice was significantly lower 40 and 60 min after intraperitoneal glucose injection compared to WT mice (Fig. 8D).

In a recent paper, Xu et al (39) showed improved glucose tolerance in P2Y\(_{14}\)-deficient mice fed with a high-fat diet due to an elevated insulin sensitivity e.g. of the liver. This is in contrast to our data where insulin sensitivity of KO and WT mice was not different (Fig. 8A/B). It was argued that reduced macrophage infiltration and hepatic inflammation account for improved hepatic insulin sensitivity (39). As in our study (Fig. 8C/D), Xu et al (39) found reduced serum insulin levels after glucose administration in KO mice. This important finding remained unappreciated in their study and other pathophysiological mechanisms were not considered. In a more reasonable scenario, lower insulin levels result in a reduced hepatic steatosis, as found in (39) under high-fat diet. In fact, the differential expression of genes involved in lipid and glycogen metabolism in liver, as found in (39), can be interpreted as a consequence of lower insulin levels. It is well known that insulin stimulates the expression of lipogenic enzymes such as fatty-acid synthase and acetyl-CoA carboxylase and inhibits the transcription of gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (40). Thus, reduced insulin secretion may also result in reduced weight gain following high caloric nutrition as seen in our P2Y\(_{14}\)-deficient mice kept under western type diet. The hepatic inflammation is most likely secondary to the alimentary hepatic steatosis found in (39). Since the western type diet we used does not significantly differ from the high-fat diet (see Experimental Procedures) Xu et al fed the mice (26.3% carbohydrates, 34.9% fat, 26.2% protein) we further focused on the reason for the lower blood insulin levels which were seen already in animals fed with standard chow.

Components involved in insulin release are differentially expressed in P2Y\(_{14}\)-deficient islets – Our functional data pointed towards a lower pancreatic insulin secretion after intraperitoneal glucose administration. High expression of P2Y\(_{14}\) was noticed in the exocrine pancreas (Figs. 3 and 4) but also in pancreatic islets (Fig. 3D). We first performed morphometric analyses in H&E and insulin immunohistochemistry stained slices in P2Y\(_{14}\)-KO mice. Neither the number nor the size of the pancreatic islets differed between WT and KO mice (Fig. 9A/C). Also, the insulin immunopositive areas were similar in both genotypes, suggesting no gross morphological abnormalities in islet development (Fig. 9B/D).

Since P2Y\(_{14}\)-deficient islets did not show obvious morphological differences we screened for those at a molecular level. Thus, mRNA expression in WT and KO pancreatic islets was analyzed in a transcriptome-wide approach using RNA-sequencing. Approximately 13 million reads per animal were analyzed. As expected, insulin, glucagon, somatostatin and SUR1/Kir6.2 transcripts were highly expressed in islets of both, WT and KO mice. In line with the data from immunohistochemistry there was no difference in insulin mRNA expression levels between WT and KO mice (suppl. Table S2). The expression of P2Y\(_{14}\) gene was significantly reduced in KO mice (suppl. Table S2), but one should note that detected P2Y\(_{14}\) transcripts in the KO islets represented mRNA sequences of the 5'UTR region and not of the coding region (Fig. 1). In line with our RNASeq data, also qPCR analysis (\(\Delta Ct = 7.64 \pm 0.55\)), \(\beta\)-galacosidase staining of pancreatic tissue (Fig. 3) and other studies (41) clearly demonstrate P2Y\(_{14}\) expression in pancreatic islets.

815 genes were found differentially expressed between WT and KO mice (329 transcripts downregulated, 486 transcripts upregulated, \(P <\)
Reduced insulin release in P2Y14-/- mice

Reduced insulin release from pancreatic islets of P2Y14-deficient mice – It is almost impossible to functionally analyze all components found to be differentially expressed between WT and KO at both, their individual level and their signaling network level. Therefore, we studied insulin secretion, as one integral islet function, in perfused and static treated islets in vitro. Although intracellular insulin content was similar in lysed islets isolated from WT and KO mice (Fig. 11A), the insulin release following stimulation with low (2.8 mM) and high glucose (16.7 mM) during a 30 min incubation was significantly reduced in islets lacking P2Y14 (Fig. 11B/C). To study the kinetics of insulin release, isolated islets were placed in a perfusion chamber and continuously perfused with KRB solution containing different glucose concentrations. The initial insulin release peak and the sustained insulin release were significantly reduced in KO islets stimulated with a high glucose concentration (16.7 mM) (Fig. 11D). These data clearly demonstrated that P2Y14 deficiency has a negative impact on glucose-dependent insulin release from isolated pancreatic islets and confirmed our in vivo findings.

Since P2Y14 couples to Gi/o proteins, inhibits adenylyl cyclases and mobilizes intracellular Ca2+ (11,28) most probably via release of Gβγ dimers from G proteins (47), one would expect that loss of this gene should rather improve glucose-mediated insulin release from β-cells. However, UDP-glucose alone was neither able to significantly release insulin nor significantly reduce glucose-induced insulin release from isolated islets (see Fig. 11). Therefore, we re-evaluated the agonistic properties of UDP- and UDP-glucose at the mouse and human P2Y14 with different methods in vitro. Techniques using chimeric G proteins in yeast and mammalian heterologous expression systems showed high basal activity of P2Y14 and some minor UDP- and UDP-glucose-mediated responses (Fig. 12A, Table 1). However, UDP and UDP-glucose did not significantly activate P2Y14 in classic cAMP inhibition assay (Table 1) and label-free DMR (Fig. 12B), whereas the Gα-coupled neuropeptide Y receptor Y2 showed robust agonist-mediated signals in all tests. At the current stage, UDP and UDP-glucose show weak agonistic activity at P2Y14 in functional assays using chimeric G proteins (2). Label-free and classic second messenger assays as well as our in vivo data with knock-out mice do not provide evidence for physiological activities of UDP and UDP-glucose at P2Y14. Further studies using clear-cut controls (e.g. gene-deficient animals) are required to finally

0.05, the complete list can be provided on request). Functional enrichment analysis of differentially expressed genes disclosed several GO (Gene Ontology) categories related to protein synthesis, signaling pathways and hormone secretion (suppl. Table S3). Since serum insulin levels were reduced in KO mice we mainly focused on genes known to be related to glucose sensing, insulin exocytosis and its modulation. Selected components involved in insulin release and their changes in transcript levels are schematically given in Figure 10.

Glucose uptake through the glucose transporter 2 (GLUT2) is a first key step in regulation of insulin release (42) (Fig. 10). The expression of GLUT2 (Slc2a2) was significantly downregulated in KO mice and may indicate a possibly reduced glucose-sensing or -responding ability of the islets.

The major trigger of insulin vesicle exocytosis is an increase in intracellular Ca2+ (42) and cAMP and protein kinase A (PKA) activity are known to amplify insulin exocytosis (43). In the classical model of GPCR-modulated insulin release, Gq (Ca2+) and Gs (cAMP) activating receptors promote insulin release, whereas Gi/o protein-coupled receptors reduce insulin release by inhibiting adenylyl cyclases and reducing cAMP levels in β-cells. Indeed, several components involved in receptor- and ion channel-controlled intracellular Ca2+ levels were downregulated in islets of KO mice, among them the subunits CACNA1C and CACNA1H of the voltage-dependent Ca2+ channels (VDCC), the endoplasmic reticulum IP3 receptor (ITPR1), cAMP-degrading phosphodiesterases PDE1C and PDE4D (44), several GPCR known to modulate insulin release such as the free-fatty acid receptor FFAR1 (45), calcium-sensing receptor (CASR), GLP1 receptor (GLP1R), GPR119, somatostatin receptors SSTR1 and SSTR2 (and their agonist somatostatin) (41,46).

In sum, transcriptome data showed significant changes in relevant components of glucose sensing, Ca2+-triggered and GPCR-modulated insulin release pathways in islets of P2Y14-deficient mice which suggested changes in islet function of KO animals.
prove or disprove UDP-Glc and UDP as physiological agonists for P2Y₁₄.

The cell type within Langerhans islets, where a specific GPCR is expressed, determines its function on insulin exocytosis. Currently, the cell type(s) of pancreatic islets expressing P2Y₁₄ is unknown. Further, the effect of a given GPCR on insulin release apparently not only depends on its G protein-coupling mode. Thus, G₃ protein-coupled receptors such as the GnRH receptor inhibits insulin release whereas the exclusively Gᵢ protein-coupled cannabinoid receptor type 1 and the melanin-concentrating hormone receptor stimulate insulin release from islets (41). The most abundant somatostatin receptor SSTR3 (see suppl. Table S2), which is Gᵢₒ protein-coupled, has no significant effect on insulin release (41). As for several GPCR expressed in pancreatic islets (41) the specific signaling pathway how P2Y₁₄ modulates gene expression and functions of islets needs to be studied further.

CONCLUSION
Mice with P2Y₁₄ deficiency present a rather mild phenotype under SPF (specific pathogen free) conditions. However, our studies revealed involvement of P2Y₁₄ in GI tract emptying and glucose homeostasis regulation. The lack of P2Y₁₄ function reduced intestine passage and glucose-stimulated insulin release from pancreatic islets in vitro and in vivo. It is of interest that several GPCR, e.g. GLP1R, GPR39, M3 muscarinic receptor and galanin receptor, modulate both, GI motility and glucose-induced insulin secretion (48-51). For P2Y₁₄ it is currently unclear whether this GPCR realizes its effect by acute activation of its signaling cascade or during islet ontogenesis. The gross transcriptome changes found in P2Y₁₄ deficient islets may also suggest a more complex alteration of the expression profile indicating a role of P2Y₁₄ in subdifferentiation of islets cells. Indeed, some genes which are involved in differentiation and maturation of pancreatic islet cell types (e.g. MAFA, MAFB, REG2) (52-54) are differentially expressed in the islets isolated from P2Y₁₄-deficient mice (Fig. 10). Although we did not find differences in gross islet morphology and islet size distribution (Fig. 9), there is evidence that e.g. pancreatic β-cells are not homogenous but rather present functionally different subpopulations with distinguishable functionality (55,56). Thus, P2Y₁₄-mediated signaling may be one signal that modulates distinct functionalities of islet cells. Since Langerhans islets derived from pancreatic ductal cells during ontogenesis and P2Y₁₄ expression was morphologically also found in the exocrine pancreas (Fig. 3), more detailed studies should focus on the relevance of P2Y₁₄ in pancreas development. Further studies with conditional β-cell-specific KO mice can help to differentiate between acute and adaptive effects of P2Y₁₄ deficiency. Nevertheless, our findings identify P2Y₁₄ as a novel modulator of insulin secretion.
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*Reduced insulin release in P2Y14<sup>−/−</sup> mice*
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Abbreviations – DMR, dynamic mass redistribution; FPKM, fragments per kilobase of transcript per million mapped reads; GI, gastrointestinal; GLUT2, glucose transporter 2; GO, Gene Ontology; GPCR, G protein-coupled receptor; KRB, Krebs-Ringer buffer; oGTT, oral glucose tolerance test; PKA, protein kinase A; SSTR1, somatostatin type 1 receptor

FIGURE LEGENDS

FIGURE 1. Generation of a P2Y14-deficient mouse strain and P2Y14 qPCR design. P2Y14-deficient mice were generated at TAKEDA Ltd.. The coding sequence was partly replaced by an ECMV-IRES / β-galactosidase cassette followed by a loxP-floxed neomycin selection cassette. We removed neomycin cassette by breeding with EIIa-Cre mice and successful removal was verified by PCR. For qPCR a primer pair matching to exon 1 and 2 was designed. Genotyping of mice was performed as described in Experimental Procedures with primers indicated as: for, rev and β-gal s.

FIGURE 2. Detection of bacterial LacZ reporter gene via β-galactosidase activity in cryosections of selected organs. To monitor the expression of P2Y14 in vivo at the cellular level a transgenic KO mouse was generated by replacement of the P2Y14–coding region with an expression cassette harboring an internal ribosome entry site and the bacterial LacZ reporter gene under control of the endogenous P2Y14 promoter. Sites of LacZ expression were located in cryosections using X-Gal, and counterstained with nuclear fast red. (A) Whereas no β-galactosidase activity was detected in WT mice, KO mice showed intensive β-galactosidase staining several other smooth muscle or contractile cell-containing organs. A indicates arterial vessel, V indicates venous blood vessel. (B) β-galactosidase staining in the GI tract was only positive in a subpopulation of smooth muscle cells. (C) The expression of P2Y14 in certain muscle layers throughout the GI tract is schematically given.

FIGURE 3. Detection of bacterial LacZ reporter gene in cryosections of pancreatic tissue. (A) Expression of LacZ reporter gene in exocrine and endocrine parts of the pancreas. The enlarged picture details show the expression in the acini (B/C) and in cells of the islet (D).

FIGURE 4. P2Y14 transcript and P2Y14-promoter-driven LacZ gene expression in mouse tissues. Total RNA was extracted from several tissues from WT and P2Y14-KO mice (n=3) and mRNA levels of either P2Y14 or LacZ transcripts were determined by SYBR Green qPCR analysis. Primers were designed for the intron-flanking exons 1 and 2 (see Fig. 1). Expression data are shown as ΔCt values normalized to the house keeping gene β2-microglobulin. (A) qPCR analysis shows a widespread receptor distribution with high expression levels in pancreas, salivary glands, brain and parts of the gastrointestinal tract and lowest expression in liver. Data shown as mean ± SEM. (B) Expression of bacterial LacZ reporter gene in

Reduced insulin release in P2Y14−/− mice

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KO mice correlates ($r^2=0.9058$) with the expression of P2Y$_{14}$ in WT mice. Dashed line indicates the 95 % confidence interval.

**FIGURE 5.** P2Y$_{14}$-deficient mice showed a delayed gastrointestinal emptying. A dye-labeled dextran was applied to mice orally and the excretion in faeces was measured hourly. The excretion of blue dextran is shown relative to the total excreted blue dextran after 8 hours. Five KO mice but only one WT animal had to be excluded from analysis according to our exclusion criterion (see Experimental Procedures). OD$_{620}$ nm ($\Delta$ 8h) = 0.8770 ± 0.0630 for WT and OD$_{620}$ nm ($\Delta$ 8h) = 0.5636 ± 0.0731 for KO mice were set to 100 % respectively. Data shown as mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t-test

**FIGURE 6.** Airway responsiveness is reduced in P2Y$_{14}$-deficient mice. Lung resistance (A) and dynamic compliance (B) were measured by invasive plethysmography (emka TECHNOLOGIES) in response to inhaled UDP-glucose and increasing doses of methacholine. Data shown as mean ± SD. Baseline values (set to 100 %) were: lung resistance 1.28 ± 0.41 mmHg x s/ml for WT mice and 1.11 ± 0.34 mmHg x s/ml for KO mice, dynamic compliance 0.84 ± 0.56 ml/mmHg for WT and 0.72 ± 0.43 ml/mmHg for KO mice; * P < 0.05; Student’s t-test.
For statistic analysis of the entire methacholine test a paired two-tailed t-test (marked by a long line) was performed (P = 0.0105 for dynamic compliance).

**FIGURE 7.** P2Y$_{14}$-deficient mice show reduced glucose tolerance. Glucose tolerance tests were performed either by oral application (A, C) of 80 mg glucose or by intraperitoneal (B, D) glucose injection (2 mg/g body weight) to overnight fasted male mice kept under standard (A, B) or western (C, D) type diet. Blood glucose levels shown as mean ± SD. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t-test.
**FIGURE 8.** P2Y$_{14}$-deficient mice showed diet-dependent lower serum insulin levels after glucose administration. First, blood glucose levels were measured in an insulin tolerance test (ITT) after intraperitoneal injection of insulin (0.75 U/kg body weight) in male mice kept under standard (A) and western (B) type diet. Blood glucose levels before insulin application (set to 100 %) were 4.9 ± 0.6 mmol/l for WT and 5.8 ± 1.0 mmol/l for KO mice fed with standard diet, and 5.6 ± 0.7 mmol/l for WT and 6.1 ± 0.9 mmol/l for KO mice kept under western type diet. Data shown as mean ± SD. * P < 0.05; Student’s t-test. Then, serum insulin levels were determined before (0 min) and 20, 40 and 60 min after intraperitoneal glucose injection (2 mg/g of body weight) in male mice kept under standard (C) and western type (D) diet. Data shown as mean ± SEM. ** P < 0.01; Student’s t-test

**FIGURE 9.** Normal architecture of pancreatic islets in P2Y$_{14}$-deficient mice. (A) Representative H&E stained pancreatic section used for morphometric analyses. (B) Insulin immunohistochemistry of representative pancreatic islets. (C) The number and area of islets in 10 serial slices of pancreatic tissue were determined using NIH ImageJ Software. The number of islets was 141 ± 17 in WT and 157 ± 11 in KO mice. (D) β-cell mass was determined as ratio of insulin positive area to total pancreatic islet area. Data shown as mean ± SD.

**FIGURE 10.** Signaling pathways involved in insulin secretion from β-cells. Several components involved in insulin release from pancreatic islets are shown. P2Y$_{14}$ deficiency led to increased (green) and decreased (red) transcription of several genes compared to islets from WT mice. Detailed data from RNASeq experiments are given in supplemental Table S2 and Table S3. A list of all differentially expressed transcripts can be provided on request.

**FIGURE 11.** Impaired insulin secretion of isolated pancreatic islets of P2Y$_{14}$-deficient mice.
For cumulative insulin measurements (A-C), pancreatic islets were isolated from WT and KO male mice and incubated 30 min with KRB solution containing 2.8 mM glucose, 16.7 mM glucose or 16.7 mM glucose plus 100 µM UDP-glucose (n=5-7). Insulin levels of lysed islets (A), in the supernatants (B) and the ratio of both (C) are shown. For kinetic insulin measurements (D), pancreatic islets were isolated from WT and KO male mice and continuously perifused with KRB solution containing 2.8 mM glucose, 16.7 mM glucose, or 16.7 mM glucose plus 100 µM UDP-glucose. Insulin levels shown as mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t-test

FIGURE 12. Testing UDP-glucose and UDP at P2Y14 in yeast and mammalian cells.
(A) Genetically engineered yeast cells (57) transformed with the indicated receptor constructs were analyzed without (basal) and with agonist (10 µM). Receptor activation was measured after 40 hours as growth of yeast cells in histidine-depleted medium by recording the optical density (OD) at 600 nm. Data (mean ± SD) from two independent experiments (in triplicate) are shown. (B) Transiently transfected HEK293 cells (P2Y14, neuropeptide Y receptor Y2) were seeded in 384-well plates the day prior DMR measurements (16). Cells were challenged with indicated concentrations of the corresponding agonist (UDP, UDP-glucose, NPY) and wavelength shift (in pm) was monitored. Each receptor stimulation curve was first corrected against own buffer control (basal) and second, against mock-transfected cells stimulated with the respective agonist. Data from three independent experiments (mean ± SEM) were summarized, each performed in quadruplicate.
TABLE 1. Functional measurements of mouse and human P2Y14 receptors.

Mouse and human P2Y14 constructs and two other Gi-coupled receptors as controls were transfected for cAMP inhibition and direct cAMP measurements (via a chimeric Gαsi5 protein) as described in Experimental Procedures. For inhibition of cAMP, transfected COS-7 cells were stimulated either with 5 μM Forskolin (FSK) or together with the indicated agonists for 15 minutes. Forskolin-stimulated values of each receptor were set 100% and % of inhibition due to the agonist stimulation was calculated for each receptor. Forskolin-stimulated cAMP values for each receptor and mock transfected cells (in nM) are given in brackets. Basal cAMP levels prior Forskolin-stimulation for mock-transfected cells were 0.88 ± 0.57 nM. The number of assays is indicated in each column. All assays were performed in triplicates.

<table>
<thead>
<tr>
<th>transfected construct</th>
<th>agonist</th>
<th>% of FSK stimulation (n=10)</th>
<th>cAMP (nM) cotransfected Gαsi5 (n=4)</th>
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<tr>
<td>mock</td>
<td>FSK/basal</td>
<td>100 (7.78 ± 1.29)</td>
<td>2.71 ± 1.42</td>
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<tr>
<td></td>
<td>UDP</td>
<td>96.93 ± 9.53</td>
<td>2.95 ± 2.22</td>
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<tr>
<td></td>
<td>UDP-Glc</td>
<td>104.06 ± 18.13</td>
<td>3.12 ± 1.18</td>
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<tr>
<td></td>
<td>NPY</td>
<td>106.08 ± 27.75</td>
<td>3.12 ± 1.44</td>
</tr>
<tr>
<td></td>
<td>CCh</td>
<td>92.13 ± 17.59</td>
<td>2.67 ± 1.76</td>
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<tr>
<td>mouse P2Y14</td>
<td>FSK/basal</td>
<td>100 (8.96 ± 1.92)</td>
<td>7.22 ± 1.99</td>
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<tr>
<td></td>
<td>UDP</td>
<td>103.37 ± 22.91</td>
<td>8.10 ± 2.18</td>
</tr>
<tr>
<td></td>
<td>UDP-Glc</td>
<td>93.81 ± 21.66</td>
<td>9.20 ± 2.05</td>
</tr>
<tr>
<td>human P2Y14</td>
<td>FSK/basal</td>
<td>100 (8.73 ± 3.12)</td>
<td>7.49 ± 3.00</td>
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<tr>
<td></td>
<td>UDP</td>
<td>99.10 ± 18.56</td>
<td>8.04 ± 3.26</td>
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<tr>
<td></td>
<td>UDP-Glc</td>
<td>98.88 ± 21.47</td>
<td>8.86 ± 3.50</td>
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<td>mouse Y2R</td>
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<td>4.18 ± 0.98</td>
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<tr>
<td></td>
<td>NPY</td>
<td>64.49 ± 15.09</td>
<td>12.10 ± 3.24</td>
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<tr>
<td>human M2R</td>
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</tr>
<tr>
<td></td>
<td>CCh</td>
<td>80.00 ± 21.28</td>
<td>11.34 ± 2.96</td>
</tr>
</tbody>
</table>
Reduced insulin release in P2Y$_{14}^{-/-}$ mice
FIGURE 2

Reduced insulin release in P2Y$_{14}^{-/-}$ mice
Reduced insulin release in P2Y14⁻/⁻ mice
Reduced insulin release in P2Y14^−/− mice

**Figure 4**

**Figure 5**

- WT n=23
- KO n=16
Reduced insulin release in P2Y$_{14}$ mice

**FIGURE 6**

A

% of baseline lung resistance

B

% of baseline dynamic compliance

**FIGURE 7**

A

Blood glucose (mmol/L) vs. time (min)

B

Blood glucose (mmol/L) vs. time (min)

C

Blood glucose (mmol/L) vs. time (min)

D

Blood glucose (mmol/L) vs. time (min)
Reduced insulin release in P2Y$_{14}^{-/-}$ mice

FIGURE 8

A

B

C

D

blood glucose (% of basal)

blood glucose (% of basal)

serum insulin (ng/ml)

serum insulin (ng/ml)

WT n=15

KO n=11

WT n=15

KO n=15

WT n=15

KO n=15

WT n=15

KO n=15

0 15 30 45 60

t (min)

0 15 30 45 60

t (min)

0 20 40 60

t (min)

0 20 40 60

t (min)
Reduced insulin release in $P2Y_{14}^{-/-}$ mice
Reduced insulin release in P2Y14<sup>−/−</sup> mice
Reduced insulin release in P2Y<sub>14</sub> <sup>−/−</sup> mice
Reduced insulin release in P2Y14−/− mice
ERKLÄRUNG ÜBER DIE EIGENSTÄNDIGE ABFASSUNG DER ARBEIT


……………………………
Datum

……………………………
Unterschrift
CURRICULUM VITAE

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EDUCATION and QUALIFICATIONS

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Endocrinology, University Clinic of Leipzig

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02/2013 Online-Tutorial “Computing (R) for Data Analysis”, John
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Doctoral scholarship of Faculty of Medicine, Leipzig

2010 Student assistant at St. Elisabeth Hospital, Leipzig
2009 Anatomy tutor at dissection course, Institute of Anatomy, Leipzig

Since 2007 Medical studies, University Leipzig
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(Abitur 1.0)

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1998 Emigration from Kazakhstan

1993 – 1998 Mathematical school, Rudnyj

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German: (almost) mother tongue
English: fluent

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Poster award: “The physiological relevance of the UDP-glucose receptor P2Y$_{14}$”, 5$^{th}$ Joint Italian-German Purine Club Meeting 2013, Italy

Scientific photo award “Fascination Microcosm” 2012, Zeiss
PUBLICATIONS

The G protein-coupled receptor P2Y14 influences insulin release and smooth muscle function in mice

The ductal origin of structural and functional heterogeneity between pancreatic islets
Prog Histochem Cytochem. 2013 Oct;48(3):103-40
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