Comparative Analyses of Murine and Human Formyl Peptide Receptor 3

Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften
der Medizinischen Fakultät
der UNIVERSITÄT DES SAARLANDES

2017

vorgelegt von: Hendrik Stempel
geb. am: 22.12.1985 in Homburg
Es gibt Stunden,
in denen der Mensch von aller
Unzulänglichkeit befreit ist.
Man steht dann auf einem
kleinen Fleck eines kleinen Planeten,
schaut erstaunt die Schönheit des Ewigen,
des in der Tiefe Unergründlichen.
Man fühlt, es gibt nicht mehr Werden und Vergehen,
es gibt nicht mehr Tod und Leben,
sondern nur das Sein.

Albert Einstein
The following manuscripts emerged from this thesis:


**Stempel H**, Zufall F, Bufe B. Evidence for an Orthologous Function of Mouse and Human Formyl Peptide Receptor 3. In preparation
# Table of Contents

List of Figures ........................................................................................................ IV  
List of Tables ........................................................................................................... VI  
Abstract .................................................................................................................. VII  
Zusammenfassung .................................................................................................... VIII  
Abbreviations .......................................................................................................... X  

## 1 INTRODUCTION .......................................................................................... 1  
1.1 Social Recognition and Olfactory Pathogen Recognition ............................ 1  
1.2 The Vomeronasal Organ ................................................................................. 3  
  1.2.1 General Function of the Vomeronasal Organ ........................................ 3  
  1.2.2 Anatomy of the Vomeronasal Organ ..................................................... 4  
  1.2.3 Detection Mechanisms in the Vomeronasal Organ ................................ 6  
  1.2.3.1 Vmn1r Expressing Vomeronasal Sensory Neurons ............................ 6  
  1.2.3.2 Vmn2r Expressing Vomeronasal Sensory Neurons ............................ 7  
  1.2.3.3 Formyl Peptide Receptor Expressing Vomeronasal Sensory Neurons ... 8  
1.3 Formyl Peptide Receptors ............................................................................. 9  
  1.3.1 General Function of Formyl Peptide Receptors .................................... 9  
  1.3.2 Genetics of Formyl Peptide Receptors ............................................... 11  
  1.3.3 Tissue Distribution of Formyl Peptide Receptors ................................ 12  
  1.3.4 Relationship between Murine and Human Formyl Peptide Receptors ... 13  
1.4 Aims of this Work ......................................................................................... 14  

## 2 EXPERIMENTAL PROCEDURES .......................................................... 15  
2.1 Ligands ......................................................................................................... 15  
  2.1.1 Synthetic Ligands .................................................................................. 15  
  2.1.2 Natural Ligands ..................................................................................... 16  
2.2 Mouse Strains ............................................................................................... 17  
2.3 Molecular Biology ......................................................................................... 17  
  2.3.1 Oligonucleotides ................................................................................... 17  
    2.3.1.1 Sequencing Primers ................................................................. 18  
    2.3.1.2 PCR Primers .............................................................................. 18  
  2.3.2 Polymerase Chain Reaction ................................................................. 19  
  2.3.3 PCR Templates ..................................................................................... 19  
  2.3.4 RNA Isolation ...................................................................................... 20  
  2.3.5 cDNA Synthesis ................................................................................... 21  
  2.3.6 Extraction of Genomic DNA ............................................................... 21  
  2.3.7 Purification of PCR Products .............................................................. 21  
  2.3.8 Gel Electrophoresis ............................................................................. 22  
  2.3.9 RNA Quantification ........................................................................... 22  
  2.3.10 Enzymatic DNA Digestion ............................................................... 22  
  2.3.11 Expression Vectors ............................................................................ 22  
  2.3.12 DNA Ligation .................................................................................... 22  
  2.3.13 Transformation of Competent Escherichia coli ................................ 24  
  2.3.14 Isolation of Plasmid DNA from Bacterial Cultures ......................... 24  
  2.3.15 Determining DNA and RNA Concentration ................................... 25  
  2.3.16 DNA Sequencing .............................................................................. 25  
  2.3.17 Generation of Bacterial Glycerol Stocks ....................................... 25  
  2.3.18 Formyl Peptide Receptor Genes ....................................................... 25  
2.4 HEK293T Cell Culture ................................................................................. 26
2.4.1 HEK293T Cells .................................................................26
2.4.2 Cell Culture Media ..........................................................26
2.4.3 Cultivating Culture Cells .....................................................26
2.4.4 Transient Transfection of Culture Cells ..................................27
2.4.5 Thawing of Cryopreserved Culture Cells .................................27
2.4.6 Storage of Culture Cells .....................................................27
2.5 High-Throughput Calcium Imaging ..........................................28
2.5.1 Cell Population Calcium Imaging ........................................28
2.5.1.1 Dye Loading of HEK293T Cells for Cell Population Calcium Imaging ......28
2.5.1.2 Data Acquisition for Cell Population Calcium Imaging with the FLIPR .....28
2.5.1.3 Analysis of FLIPR Experiments ........................................28
2.5.2 Single Cell Calcium Imaging ............................................29
2.5.2.1 Dye Loading of HEK293T Cells for Single Cell Calcium Imaging ..........29
2.5.2.2 Data Acquisition for Single Cell Calcium Imaging with the Bioimager ....30
2.5.2.3 Analysis of Bioimager Experiments ....................................30
2.6 Immunocytochemistry ..........................................................31
2.6.1 Preparation of Samples for Immunocytochemistry .....................31
2.6.1.1 Dissociation of Vomeronasal Tissue ..................................31
2.6.1.2 Preparation of Blood Cells .............................................31
2.6.1.3 Preparation of Bone Marrow Cells ....................................31
2.6.2 Immunostaining Protocol ................................................32
2.6.2.1 Image Acquisition and Data Analysis for General Immunostainings .....32
2.6.3 Antibodies .....................................................................32
2.6.3.1 Used Antibodies ...........................................................32
2.6.3.2 Generation of Fpr3 Antibodies .........................................33
2.6.3.3 Peptide-Spot Array Analysis for Antibody Characterization ..........34
2.6.3.4 Blocking Peptides ......................................................34
2.7 In Situ Hybridization ............................................................35
2.7.1 Coronal Slices of the Vomeronasal Organ for In Situ Hybridization .......35
2.7.2 Design and Generation of RNA Probes ..................................35
2.7.3 Hybridization ...............................................................36
2.7.4 Washing ..................................................................36
2.7.5 Detection with Alkaline Phosphatase ....................................36
2.8 Software and Web Tools ..........................................................37
2.8.1 Software ..................................................................37
2.8.1.1 Adobe Photoshop CS5 ..................................................37
2.8.1.2 Adobe Illustrator CS6 ...................................................37
2.8.1.3 Microsoft Office 2010 ....................................................37
2.8.1.4 VectorNTI Suite 9 ..........................................................37
2.8.1.5 FLIPR system software v2.1.2 ........................................37
2.8.1.6 BD AttoVision™ software v1.6 .......................................37
2.8.1.7 Graph Pad Prism .........................................................38
2.8.1.8 Cell® P ..................................................................38
2.8.2 Web Tools .................................................................38
2.8.2.1 Gene Information Gathering .........................................38
2.8.2.2 Prediction of Gene Orthology .......................................38
2.8.2.3 Prediction of Transmembrane Domains (TMHMM) ..................39
2.8.2.4 Calculation of Primer Melting Temperatures ........................39
2.8.2.5 Mouse Haplotype Analyses ..........................................39
2.9 Statistics and Mathematics ......................................................39
2.9.1 Average and Standard Deviation ........................................39
3 RESULTS.......................................................................................................................... 41
3.1 Generation and Characterization of Two Novel Fpr3 Antibodies................................. 41
  3.1.1 Characterization of Commercially Available Fpr3 Antibodies............................... 41
  3.1.2 Generation of Fpr3 Antibodies............................................................................... 42
  3.1.2.1 Generation of Polyclonal Rabbit Fpr3 Antibodies........................................... 43
  3.1.2.2 Generation of Monoclonal Mouse Fpr3 Antibodies........................................... 44
  3.1.3 Characterization of Two Novel Fpr3 Antibodies.................................................... 46
3.2 Fpr3 Expression in the Murine Vomeronasal and Immune Systems......................... 49
  3.2.1 Fpr3 Protein is Expressed in Vomeronasal Sensory Neurons and in Immune Cells......................................................................................................................... 49
  3.2.2 Fpr3 Expression in Neutrophil Granulocytes is Enhanced by LPS Stimulation..... 53
3.3 Strain-Specific Variants of Murine Fpr3.................................................................... 58
  3.3.1 Fpr3 Protein Expression Occurs in a Strain-Specific Manner ............................ 58
  3.3.2 Loss of Fpr3Δ424-435 Function Due to Diminished Receptor Expression....... 61
  3.3.3 Lack of Fpr3Δ424-435 Expression in HEK Cells.................................................. 63
  3.3.4 Fpr3Δ424-435 is Non-Functional in the Vomeronasal Organ......................... 65
3.4 Comparative Characterization of Murine and Human Fpr3 Function..................... 66
  3.4.1 Orthology between Murine and Human Fpr3 is not Assessable by Sequence Comparison ......................................................................................................................... 67
  3.4.2 Murine and Human Fpr3 Show Similar Functional Properties.......................... 67
  3.4.3 Murine and Human Fpr3 are More Selective than Fpr1 and Fpr2............... 69
  3.4.4 Specification of Fpr3 Function in Mouse and Human............................................. 71
  3.4.5 Ligand Preferences of Murine and Human Fpr3 Differ Partially......................... 73
4 DISCUSSION...................................................................................................................... 76
4.1 Fpr3 of Mouse and Human are Functional Orthologs........................................... 77
  4.1.1 Genetic Evidence for the Orthology between Fpr3 of Mouse and Human......... 77
  4.1.2 Fpr3 of Mouse and Human Share Functional Similarities.................................... 78
  4.1.3 Adaptations of Murine Fpr3 for a Function in Olfaction...................................... 79
4.2 Murine Fpr3 is Expressed in Multiple Tissues....................................................... 80
  4.2.1 Fpr3 Protein is Expressed in the Vomeronasal Organ.......................................... 80
  4.2.2 Fpr3 Protein is Expressed in Immune Cells......................................................... 82
4.3 Strain-Specific Fpr3 Variants .................................................................................. 83
  4.3.1 Two Functionally Distinct Fpr3 Variants Exist in Mice....................................... 84
  4.3.2 Truncation of Fpr3Δ424-435 Protein Causes a Lack of Receptor Function........ 85
  4.3.3 Distribution of the Fpr3 Variants Amongst Laboratory Mice............................ 85
  4.3.4 Fpr3 Variants Originated in Wild Living Mice..................................................... 88
4.4 Outlook.................................................................................................................... 89
5 LITERATURE...................................................................................................................... 91
Appendix ............................................................................................................................. 104
Copyright Permission Policy of the American Society for Biochemistry and Molecular Biology
Einverständniserklärung
Acknowledgements
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Olfactory perception is crucial for sick conspecific aversion</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Organization of the murine vomeronal organ</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Receptors expressed in the vomeronal organ</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Signal peptide structure</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>Relationship and genomic organization of human and murine Fpr genes</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>Schemata of the expression cassettes of pcDNA™3.1 (+) and pcDNA™5/FRT/TO</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Schema of the expression cassette of pcDNA™5/FRT/TO for Fpr3Δ424-435</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>Representative responses in FLIPR experiments</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>Test of the commercially available Fpr3 antibody N-20</td>
<td>41</td>
</tr>
<tr>
<td>10</td>
<td>Characterization of the commercially available Fpr3 antibody M-20</td>
<td>42</td>
</tr>
<tr>
<td>11</td>
<td>Epitopes of the commercially available Fpr3 antibody M-20</td>
<td>44</td>
</tr>
<tr>
<td>12</td>
<td>Characterization of monoclonal mouse Fpr3 antibodies</td>
<td>45</td>
</tr>
<tr>
<td>13</td>
<td>Epitopes of the two novel specific Fpr3 antibodies ECL1 and ECL2</td>
<td>47</td>
</tr>
<tr>
<td>14</td>
<td>ECL1 and ECL2 detect Fpr3 and do not recognize other murine Fpr family members</td>
<td>48</td>
</tr>
<tr>
<td>15</td>
<td>ECL1 and ECL2 show high sensitivity for Fpr3</td>
<td>49</td>
</tr>
<tr>
<td>16</td>
<td>Fpr3 mRNA is expressed in the vomeronal organ</td>
<td>50</td>
</tr>
<tr>
<td>17</td>
<td>Fpr3 protein is expressed by a small subset of vomeronal cells</td>
<td>50</td>
</tr>
<tr>
<td>18</td>
<td>Characterization of Fpr3-positive vomeronal cells</td>
<td>52</td>
</tr>
<tr>
<td>19</td>
<td>Molecular characteristics of the non-olfactory Fpr3-positive cells</td>
<td>53</td>
</tr>
<tr>
<td>20</td>
<td>Fpr3 is expressed in mouse leukocytes</td>
<td>54</td>
</tr>
<tr>
<td>21</td>
<td>Fpr3 protein is expressed in mouse neutrophil granulocytes</td>
<td>54</td>
</tr>
<tr>
<td>22</td>
<td>Fpr3 mRNA is absent in blood leukocytes</td>
<td>55</td>
</tr>
<tr>
<td>23</td>
<td>Fpr3 mRNA expression is induced by LPS stimulation</td>
<td>56</td>
</tr>
<tr>
<td>24</td>
<td>Human neutrophil granulocytes express three Fprs</td>
<td>57</td>
</tr>
<tr>
<td>25</td>
<td>Fpr3 protein levels rise with increasing mRNA levels upon LPS stimulation</td>
<td>57</td>
</tr>
<tr>
<td>26</td>
<td>Strain-specific loss of Fpr3 expression in mice</td>
<td>59</td>
</tr>
<tr>
<td>27</td>
<td>Sequence and distribution of the 12 nucleotide in-frame deletion in Fpr3Δ424-435</td>
<td>60</td>
</tr>
</tbody>
</table>
Figure 28. Fpr3Δ424-435 does not respond to the synthetic Fpr3 agonist W-peptide  
Figure 29. Fpr3Δ424-435 does not respond to natural Fpr3 activators  
Figure 30. Expression of Fpr3Δ424-435 and Fpr3wt in HEK293T cells  
Figure 31. Fpr3Δ424-435 exhibits truncated expression in HEK293T cells  
Figure 32. Fpr3 agonists activate knobs of VSNs  
Figure 33. Sequence relationship between murine Fpr3 and human Fprs  
Figure 34. Murine and human Fpr3 show related agonist responses  
Figure 35. Mouse and human Fpr3 display similar agonist selectivity  
Figure 36. Mouse and human Fpr3 differ in their agonist sensitivity  
Figure 37. Calcium responses of murine and human Fpr3 saturate at 30 µM ligand concentration  
Figure 38. The ligand recognition of murine and human Fpr3 partially differs  
Figure 39. Fpr3-positive leukocytes contain polymorphonuclear nuclei  
Figure 40. Distribution of the Fpr3 variants in the laboratory mouse genealogy using the example of Castle’s mice
List of Tables

Table 1. Synthetic ligands used in this study 15
Table 2. Natural ligands used in this study 16
Table 3. Used mouse strains 17
Table 4. Sequencing primers 18
Table 5. PCR primers 18
Table 6. PCR templates 20
Table 7. Cell culture media 26
Table 8. Antibodies used in this study 33
Table 9. Peptide fragments for the generation of monoclonal Fpr3 antibodies 46

Appendix

Table 10. Peptide spot array epitopes 104
Table 11. Mouse genes containing the AMKEK motif 105
Table 12. Mouse genes containing the LNTA motif 105
Table 13. Haplotype analysis of the Fpr3 gene in different mouse strains 107
Abstract

Formyl peptide receptors (Fprs) are important and broadly tuned G protein-coupled pathogen-sensors. The murine and human Fpr gene families are comprised of seven and three genes, respectively. Fpr1 and Fpr2 of both species have been studied extensively, but the functions of murine and human Fpr3 are poorly understood. This study provides new insight into the expression and function of murine Fpr3 in the vomeronasal and immune systems and reports the existence of natural knockout strains of this receptor.

A key result of this thesis is the discovery of a large panel of distinct mouse strains that exhibit severely altered Fpr3 expression and function. Two Fpr3 receptor variants, such as Fpr3\textsubscript{wt} and Fpr3\textsubscript{Δ424–435}, which showed distinct expression patterns, were identified using two newly generated Fpr3-specific antibodies. Thereby, a lack of receptor expression was attributed to a 12 nucleotide in-frame deletion in the Fpr3\textsubscript{Δ424–435} gene. The lack of four amino acids produced an unstable and truncated Fpr3\textsubscript{Δ424–435} receptor protein. In line with these findings, calcium imaging in an in vitro expression system and in dendritic endings of vomeronasal sensory neurons showed a lack of function for this receptor variant. Fpr3\textsubscript{Δ424–435} was present in at least 19 mouse strains, whereas Fpr3\textsubscript{wt} was encoded in at least 13 other strains. These data suggest a large number of mouse strains with no known Fpr3 function. The discovery of a multitude of natural Fpr3 knockout mouse strains will be valuable to study murine Fpr3 function in the context of various genetic backgrounds.

A second key finding is the dual detection of murine Fpr3 protein expression in chemosensory neurons of the olfactory system and in specific immune cells. Significant sequence overlap and common expression in immune cells suggested a similar biological role for murine and human Fpr3. In addition, comparative in vitro calcium imaging experiments showed that the functional properties of murine and human Fpr3 are similar but differed drastically from those of Fpr1 and Fpr2. Thereby, Fpr3 of both species shared strongly overlapping agonist response patterns. These data provide clear evidence that murine and human Fpr3 are functional orthologous genes. However, concentration-response curves and structural derivative testing of a typical Fpr agonist revealed subtle tuning differences between murine and human Fpr3. Taken together, these data suggest a similar biological role for human and mouse Fpr3 with subtle functional adaptations in murine Fpr3 for olfactory system requirements.
Zusammenfassung

Formylpeptidrezeptoren (Fprs) sind wichtige G-Protein gekoppelte Pathogensensoren, die ein breites Ligandenspektrum aufweisen. Die Fpr-Genfamilie der Maus umfasst sieben Gene, während die des Menschen aus drei Genen besteht. Fpr1 und Fpr2 beider Spezies sind weitreichend erforscht, allerdings ist die Funktion des murinen und menschlichen Fpr3 unzureichend verstanden. Diese Arbeit liefert neue Erkenntnisse über die Expression und Funktion des Fpr3 der Maus im Vomeronasalorgan und Immunsystem, und zeigt das Vorkommen natürlicher Knockout-Stämme für diesen Rezeptor.


funktionellen Anpassungen des murinen Fpr3 an Anforderungen des olfaktorischen Systems hin.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NH₂</td>
<td>functional amino group (−NH₂) at the C-terminus of a protein</td>
</tr>
<tr>
<td>+RT</td>
<td>reverse transcription reaction with reverse transcriptase</td>
</tr>
<tr>
<td>-RT</td>
<td>reverse transcription control without reverse transcriptase</td>
</tr>
<tr>
<td>A₂₆₀</td>
<td>absorption at a wavelength of 260 nm</td>
</tr>
<tr>
<td>A₂₈₀</td>
<td>absorption at a wavelength of 280 nm</td>
</tr>
<tr>
<td>Aβ₄₂</td>
<td>amyloid-beta 42</td>
</tr>
<tr>
<td>Ac²-2₆</td>
<td>annexin/lipocortin 1-mimetic peptide</td>
</tr>
<tr>
<td>AOB</td>
<td>accessory olfactory bulb</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ATG</td>
<td>start codon</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BGH</td>
<td>bovine growth hormone poly-adenylation site</td>
</tr>
<tr>
<td>Bioimager</td>
<td>Bioimager BD Pathway 855</td>
</tr>
<tr>
<td>Cl</td>
<td>physiological salt solution category 1</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CD₄₅R</td>
<td>R-isoform of the cluster of differentiation molecule 45</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus promoter sequence</td>
</tr>
<tr>
<td>Co.</td>
<td>company</td>
</tr>
<tr>
<td>CT</td>
<td>C-terminus</td>
</tr>
<tr>
<td>cursive lettering</td>
<td>gene (≠ normal lettering that describes a protein)</td>
</tr>
<tr>
<td>dATP</td>
<td>2′-deoxyadenosine 5′-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2′-deoxycytidine 5′-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl dicarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2′-deoxyguanosine 5′-triphosphate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DIOPT</td>
<td>DRSC Integrative Ortholog Prediction Tool</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>2′-deoxynucleoside 5′-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>2′-deoxythymidine 5′-triphosphate</td>
</tr>
</tbody>
</table>
DTT  dichlorodiphenyltrichloroethane
EC₅₀  half maximal effective concentration
ECL  extracellular loop
EDTA  ethylenediaminetetraacetic acid
ESP  exocrine gland-secreting peptide
f-  functional formyl group (-CHO) at the N-terminus of a protein
ΔF  maximal change in fluorescence
F₀  baseline fluorescence
F_max  fluorescence maximum
F_min  fluorescence minimum
FCS  fetal calf serum
FL#  internal laboratory identifier number for ligands
FLIPR  fluorometric imaging plate reader
Fpr  (murine) formyl peptide receptor
FPR  human formyl peptide receptor
Fpr3Δ424-435  non-functional strain-specific receptor variant of (murine) formyl peptide receptor 3 containing a 12 nucleotide in-frame deletion from nucleotide 424 to 435
Fpr3_wt  functional strain-specific receptor variant of (murine) formyl peptide receptor 3
Fpr-rs  formyl peptide receptor-related sequence
FuLe  full length
g  acceleration due to gravity
Gapdh  glyceraldehyde 3-phosphate dehydrogenase
Gα₁₆  G protein alpha 16 subunit
Gα₂  G protein alpha i2 subunit
Gαₒ  G protein alpha o subunit
gene ID  gene identification number
Gγ₂  G protein gamma 2 subunit
Gγ₈  G protein gamma 8 subunit
h  hour
H₂-Mv  gene encoding a major histocompatibility complex molecule
HBSS  Hank’s balanced salt solution
HEK  human embryonic kidney
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>293T</td>
<td>cell line 293 that expresses the simian virus 40 large T antigen</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid</td>
</tr>
<tr>
<td>hFPR</td>
<td>human formyl peptide receptor</td>
</tr>
<tr>
<td>HGNC</td>
<td>HUGO Gene Nomenclature Committee</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus 1</td>
</tr>
<tr>
<td>hND</td>
<td>N-terminus of human NADH-ubiquinone oxidoreductase chain</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus derived fusion sequence</td>
</tr>
<tr>
<td>HSV-tag</td>
<td>11 amino acids derived from herpes simplex virus glycoprotein D</td>
</tr>
<tr>
<td>ICL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LL-37</td>
<td>human antimicrobial peptide LL-37</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Ly6G</td>
<td>lymphocyte antigen 6G</td>
</tr>
<tr>
<td>mATP6</td>
<td>N-terminus of murine ATP synthase A chain protein 6</td>
</tr>
<tr>
<td>mCOIII</td>
<td>N-terminus of murine Cytochrome c oxidase polypeptide III</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>mCytb</td>
<td>N-terminus of murine Cytochrome b</td>
</tr>
<tr>
<td>mFpr</td>
<td>murine formyl peptide receptor</td>
</tr>
<tr>
<td>MGI</td>
<td>mouse genome informatics</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MHV</td>
<td>mouse hepatitis virus</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>M. m.</td>
<td><em>Mus musculus</em></td>
</tr>
<tr>
<td>mND</td>
<td>N-terminus of murine NADH-ubiquinone oxidoreductase chain</td>
</tr>
<tr>
<td>MOB</td>
<td>main olfactory bulb</td>
</tr>
<tr>
<td>MOE</td>
<td>main olfactory epithelium</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MUP</td>
<td>major urinary protein</td>
</tr>
<tr>
<td>n</td>
<td>number of performed experiments</td>
</tr>
<tr>
<td>N-</td>
<td>N-terminus of a protein</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NCBI</td>
<td>national center for biotechnology information</td>
</tr>
</tbody>
</table>
OMP  olfactory marker protein
Order#  order number
ψ  pseudogene
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
PDE4A  phosphodiesterase 4A
PH#  internal laboratory identifier number for primers
ppi  pixels per inch
PVDF  polyvinylidene difluoride
RFU  relative fluorescence units
Rho  rhodopsin-derived fusion sequence
Rho-Fpr3Δ424-435-HSV  fusion protein of the Fpr3Δ424-435 receptor variant with an
  N-terminal Rho-tag and a C-terminal HSV-tag
Rho-Fpr3wt  fusion protein of the Fpr3wt receptor variant with an N-terminal
  Rho-tag
Rho-tag  the first 39 amino acids of bovine rhodopsin
RNA  ribonucleic acid
rpm  revolutions per minute
RPMI-1640  Roswell Park Memorial Institute medium
RT  room temperature
RT-PCR  reverse transcription polymerase chain reaction
S  septum
S.D.  standard deviation
SDS  sodium dodecyl sulfate
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC  super optimal broth with catabolite repression
SP  signal peptide
Sp6  Sp6 bacteriophage growing on Escherichia coli
SPase  signal peptidase
SSC  saline-sodium citrate
SV40  simian virus 40
T2R16-HSV  fusion protein of the human type 2 taste receptor 16 and a
  C-terminal HSV-tag
T7  T7 bacteriophage growing on Salmonella typhimurium
TASP  | thermosensitive alkaline phosphatase
---|---
TE  | Tris-EDTA
TGA | stop codon
TMHMM | transmembrane helix prediction based on a Markov model
TN  | Tris-HCl, NaCl
TO  | tetracycline operator
Tris | 2-amino-2-(hydroxymethyl)propane-1,3-diol
U  | enzyme unit
Vmn1r, V1r | vomeronasal type 1 receptor
Vmn2r, V2r | vomeronasal type 2 receptor
Vmn2r+ H2-Mv+ | vomeronasal type 2 receptor coexpressed with a $H2-Mv$ gene
Vmn2r+ H2-Mv− | vomeronasal type 2 receptor not coexpressed with a $H2-Mv$ gene
VNO | vomeronasal organ
VSN | vomeronasal sensory neuron
YT | yeast extract and tryptone


1 INTRODUCTION

1.1 Social Recognition and Olfactory Pathogen Recognition

Important information about the social status of conspecifics is conveyed by olfactory cues in rodents (Restrepo et al., 2006; Chamero et al., 2007; Brennan, 2009; Li et al., 2013). Chemical signals used to transmit information between conspecifics are commonly called pheromones. Pheromones are substances secreted to the outside by an individual and received by another individual of the same species that trigger distinct reactions, such as behaviors or developmental processes (Karlson and Lüscher, 1959).

The first ever described pheromone-mediated effect in mammals was the “Whitten effect”. In 1956, Wesley Whitten observed the induction of estrus in anestrus female mice upon exposure to male pheromones (Whitten, 1956). Soon after, the “Bruce effect” was demonstrated by Hilda Bruce in 1959 (Bruce, 1959). She described that newly mated female mice experience a pregnancy block and return to estrus if exposed to unfamiliar males prior to implantation of the embryo. A third mammalian pheromone effect that communicates the social status of an animal was discovered in 1969 by John Vandenbergh (Vandenbergh, 1969). The “Vandenbergh effect” portrays the acceleration of puberty in female mice upon exposure to male chemosignals. Interestingly, a delay in puberty could be induced by exposure to female pheromones (Cowley and Wise, 1972; Vandenbergh, 1973; Novotny et al., 1986; Jemiolo and Novotny, 1994). Since then, many other important social behavioral effects induced by olfactory cues have been reported, including sexual behavior and preference, parental behavior, and discrimination of individuals (Jemiolo et al., 1991; Leypold et al., 2002; Haga et al., 2010), parental behavior (Brouette-Lahlou et al., 1999; Numan et al., 2006), aggression (Stowers et al., 2002; Chamero et al., 2011), and the discrimination of individuals (Hurst et al., 2001; Restrepo et al., 2006; Kelliher et al., 2006).

Despite the many benefits of social behaviors, close interactions among conspecifics increase the risk of parasite transmission and pathogenic infection (Altizer et al., 2003; Kavaliers et al., 2004). To prevent these events, mice and other rodents are able to recognize infected conspecifics and display aversive responses to contagious individuals. Behavioral and genetic evidence suggests the involvement of the sense of smell in this process (Penn and Potts, 1998; Kavaliers et al., 2004). The best studied system is the health status-dependent choice of mating partners. In 1982, William Hamilton and Marlene Zuk first suggested that animals examine urine and fecal cues to identify disease and parasite-free mates (Hamilton and Zuk, 1982). Mice preferentially select parasite-free or parasite-resistant mating partners.
INTRODUCTION

(Hamilton and Zuk, 1982; Kavaliers et al., 2000; Ehman and Scott, 2002). Female mice were shown to discriminate odors from healthy males against others infected with different parasites, such as nematodes, (Kavaliers and Colwell, 1995; Ehman and Scott, 2001; Kavaliers et al., 2003a), protozoans (Kavaliers and Colwell, 1993; Kavaliers et al., 1997), influenza virus (Penn et al., 1998), or lice (Lehmann, 1993; Kavaliers et al., 2003b). In all cases, the female mice actively avoided infected males or their odors (Kavaliers et al., 2005). Male mice also showed aversion to urine odors of other males infected with parasites (Kavaliers et al., 2004). They refused to copulate with females infected with nematodes (Edwards and Barnard, 1987) and displayed aversive behavior to them (Gourbal and Gabrion, 2004) or their odors (Kavaliers et al., 1998). Resistance to the nematode Heligomosomoides polygyrus is heritable (Wahid et al., 1989). Thus, this odor-based mate selection is considered an indicator of “good genes” (Kavaliers et al., 2005).

Although many social interactions based on odors of infected conspecifics are known, the underlying molecular and cellular basis for perceiving an olfactory pathogen is unclear. The olfactory system of most mammals is subdivided into two principal subsystems, the main olfactory and the vomeronasal systems, which both trigger innate avoidance behaviors (Kobayakawa et al., 2007; Papes et al., 2010). Furthermore, two other olfactory structures, such as the septal organ of Masera and the Grueneberg ganglion, also play roles detecting pheromones and pheromone-driven behaviors (Roppolo et al., 2006; Ma, 2007; Tirindelli et al., 2009). One study provided clear evidence for involvement of the murine vomeronasal organ (VNO) mediating the avoidance of sick conspecifics (Boillat et al., 2015). The authors demonstrated a preference of mice for healthy conspecifics over mice that were injected with lipopolysaccharide (LPS). LPS is an endotoxin produced by Gram-negative bacteria that activates the immune system and mimics a bacterial infection (Dantzer et al., 2008). They also described aversive behavior to urine of mice infected with the mouse hepatitis virus (MHV) (Boillat et al., 2015). Mice without a functional VNO showed no aversion (Figure 1). Hence, the VNO may function as a sensor of infection.

In line with the idea that the VNO is involved in pathogen sensing, odors from sick animals activate neurons in the vomeronasal pathway of wild-type mice (Boillat et al., 2015). However, mice without a functional VNO lack this activation (Boillat et al., 2015). In line with these experiments, sensory cells in the VNO detect inflammatory markers and bacterial peptides, which are typical activators of formyl peptide receptors (Fprs) found in the immune system (unpublished data). Furthermore, several members of the murine Fpr gene family are
expressed in the VNO (Liberles et al., 2009; Rivière et al., 2009). Thus, Fprs expressed in the VNO are prime candidates for olfactory pathogen sensing.

![Figure 1](image.png)

Figure 1. **Olfactory perception is crucial for sick conspecific aversion.** Mice with an intact VNO (upper right) avoid mice or urine infected with LPS or MHV (left). Mice with a non-functional VNO (lower right) show no aversion. Figure was adapted from Boillat et al., 2015.

1.2 The Vomeronasal Organ

1.2.1 General Function of the Vomeronasal Organ

The VNO is the receptor organ of the accessory olfactory system. It contributes to social recognition and chemical communication – processes that are mediated by pheromones (Halpern and Martínez-Marcos, 2003). The crucial function of the VNO as a pheromone sensing system is well-established. Several studies examined mice after removal of the organ and reported the absence of typical pheromone-driven effects, such as the associated estrus induction (Whitten effect) (Sánchez-Criado and Gallego, 1979; Sánchez-Criado, 1982; Mora and Cabrera, 1997), pregnancy block (Bruce effect) (Reynolds and Keverne, 1979; Bellringer et al., 1980), and puberty acceleration (Vandenbergh effect) (Lomas and Keverne, 1982). All these effects were still observable in control mice that possessed a functional VNO. Pheromone-driven effects and behaviors were also attenuated by ablation of different genes expressed in the VNO. Tissue-specific deletion of signal transduction elements, such as genes encoding the G protein alpha o subunit (Gαo), the G protein gamma 8 subunit (Gγ8), or a family of nine nonclassical class I major histocompatibility complex (MHC) genes, the H2-Mv genes, severely reduced aggression (Chamero et al., 2011; Montani et al., 2013; Leinders-Zufall et al., 2014). Additionally, Gαo gene removal impaired a wide range of reproductive pheromone-regulated behaviors in adult mice, including estrus induction
(Whitten effect) and puberty acceleration (Vandenbergh effect) (Oboti et al., 2014). Furthermore, mice deficient for Trpc2, another essential signal transduction component for VNO function, showed striking behavioral defects in regulating a wide range of sexual and social behaviors (Zufall, 2005; Zufall et al., 2005). In summary, these findings show that the VNO is a key element for pheromone perception.

An important function of the VNO beyond pheromone recognition is the detection of predator odors (Papes et al., 2010; Isogai et al., 2011). Specific substances contained in these odors, so-called kairomones, elicit powerful fear-like reactions in prey species that facilitate escape, induce freezing and avoidance, and can increase stress hormone levels (Apfelbach et al., 2005). Known kairomones include trimethyl-thiazoline, a volatile component of fox odor (Kobayakawa et al., 2007), several lipocalins found in rat urine and cat saliva (Papes et al., 2010), and 2-phenylethylamine that is produced in many carnivore urines (Ferrero et al., 2011). Kairomones can derive from different odor sources, such as urine, feces, saliva, and fur. Interestingly, different kairomones are detected by the VNO and the main olfactory epithelium and detection mechanisms may depend on stimulus volatility (Liberles, 2014).

In line with this observation, growing evidence emerges for the contribution of the main olfactory epithelium to the detection of social chemosignals (Spehr et al., 2006a). Some non-volatile chemosignals established earlier as sensory stimuli of the VNO were shown to activate cells in the main olfactory epithelium (Ziesmann et al., 2002; Lin et al., 2004; Xu et al., 2005; Spehr et al., 2006b). Hence, the main olfactory epithelium is also involved in mediating social behaviors. In line with these thoughts, impairment of sexual behavior in mice lacking a functional main olfactory epithelium but possessing an intact VNO provided clear evidence for the involvement of the main olfactory epithelium in social behaviors (Keller et al., 2006). However, behavioral tests using mice with genetic and surgical lesions showed that stimulation of each system by the same substance can result in differing behavioral effects. This suggests that the same chemosignals can convey distinct social behaviors through differential activation and processing through different olfactory systems (Spehr et al., 2006a; Pérez-Gómez et al., 2014).

1.2.2 Anatomy of the Vomeronasal Organ

The VNO was first described in 1813 by Danish anatomist Ludwig Jacobson (Jacobson, 1813). It is a sensory organ that is present in most mammals (Liberles, 2014). Anatomically, the VNO consists of a pair of bilateral symmetrical cylinders. It is located at the base of the anterior nasal septum. There, it is protected by a boney capsule that is formed by the vomer
INTRODUCTION

bone. On the posterior side it ends blindly, whereas on the anterior side it is connected with the nasal cavity via a water-filled duct (Broman, 1920). Substances can enter the nasal cavity through sniffing (Halpern, 1987; Dulac and Torello, 2003; Liberles, 2014). Vomeronasal agonists resolved in fluids have to be sucked actively into the nasal cavity. An active pumping mechanism generated by pulsing blood vessels located laterally of the lumen in the non-sensory epithelium creates a suction that transports the substances through the lumen to the crescent-shaped vomeronasal sensory epithelium (Figure 2) (Døving and Trotier, 1998). The vomeronasal sensory epithelium houses vomeronasal sensory neurons (VSNs). These are specialized nerve cells express distinct receptors that detect pheromone substances (Brennan and Zufall, 2006).

Figure 2. Organization of the murine vomeronasal organ. Midsaggital view of the nasal cavity and forebrain. Sensory neurons in the main olfactory epithelium (MOE) project their axons to glomeruli in the main olfactory bulb (MOB). Vomeronasal sensory neurons (VSNs) in the apical (red) or basal (green) layer of the vomeronasal organ (VNO), which is located at the base of the nasal septum (S), project to the anterior (red) or posterior (green) side of the accessory olfactory bulb (AOB). Figure was adapted and modified from Zufall et al., 2005.

In 1970 the segregation of axon projections to the main olfactory bulb and the accessory olfactory bulb, specialized anterior brain areas, was documented in rodents (Winans and Scalia, 1970). The data suggested two parallel projection pathways to the brain, one for the VNO and one for the main olfactory epithelium (Raisman, 1972; Scalia and Winans, 1975). More than 20 years later, the separate projection of VSNs to the accessory olfactory bulb could be demonstrated by tracking axons with histological markers (Jia and Halpern, 1996; Belluscio et al., 1999; Rodriguez et al., 1999). Vomeronasal sensory neurons project to the accessory olfactory bulb (Figure 2). This structure serves as a first processing center for vomeronasal information (Munger et al., 2009). On the molecular level the vomeronasal sensory epithelium can be subdivided into an apical and a basal layer (Halpern et al., 1995; Dulac and Axel, 1995; Berghard and Buck, 1996). VSNs from different VNO layers project to different sides of the accessory olfactory bulb. VSNs located in the apical layer project to six
to 30 small glomeruli in the rostral half of the accessory olfactory bulb, whereas basal VSNs project to a similar amount of small glomeruli in the posterior half of the accessory olfactory bulb (Jia and Halpern, 1996; Belluscio et al., 1999; Rodriguez et al., 1999; Del Punta et al., 2002a). From there, mitral cells interconnect the accessory olfactory bulb with higher brain regions, such as the amygdala and hypothalamic nuclei (Halpern, 1987).

1.2.3 Detection Mechanisms in the Vomeronasal Organ

Two main populations of VSNs are anatomically segregated into an apical and a basal zone that together form the vomeronasal sensory epithelium. These main populations differ in their expressed receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997) and down-stream signal transduction molecules (Dulac and Axel, 1995; Halpern et al., 1995; Berghard and Buck, 1996).

The VNO detects many substances from different chemical classes (Zufall and Leinders-Zufall, 2007). Its ligand spectrum is complex and reaches from volatile substances to non-volatile peptides. Details are still under investigation, but the current view assigns detection of different ligand classes to specific zones of the vomeronasal sensory epithelium. VSNs of the apical zone expressing vomeronasal type 1 receptors (Vmn1rs) detect volatile substances, whereas basal VSNs expressing vomeronasal type 2 receptors (Vmn2rs) are responsible for the detection of peptide ligands (Liberles, 2014).

1.2.3.1 Vmn1r Expressing Vomeronasal Sensory Neurons

VSNs expressing Vmn1rs represent the vast majority of the apical VNO layer (Dulac and Axel, 1995; Pantages and Dulac, 2000; Leinders-Zufall, 2000). Vmn1rs are coexpressed with the G protein alpha i2 subunit (Gαi2) (Berghard and Buck, 1996; Jia and Halpern, 1996) and the G protein gamma 2 subunit (Gγ2) (Rünnenburger et al., 2002). In mouse, 191 intact of a total of 308 Vmn1r genes are known (Zhang et al., 2007).

Vmn1r expressing cells can detect small volatile substances (Leinders-Zufall, 2000; Zufall and Leinders-Zufall, 2007). Electrovomeronosagrams revealed activation of apical VSNs through substances, such as 2,5-dimethylpyrazine and 2-heptanone (Leinders-Zufall, 2000; Del Punta et al., 2002b). Consistent with the notion that Vmn1rs are activated by volatiles, knockout of a ~600 kb genomic region that contains a cluster of 16 intact Vmn1r genes resulted in impaired recognition for volatiles, such as n-pentyl acetate and isobutylamine (Del Punta et al., 2002b). However, this knockout did not give information about which of the deleted receptors were responsible for the perception of these substances. Further studies
addressing this question reported three specific receptor-ligand interactions for Vmn1rs (Boschat et al., 2002; Haga-Yamanaka et al., 2014; Haga-Yamanaka et al., 2015). One study assigned the recognition of 2-heptanone to the receptor V1r2b by functional experiments on vomeronasal sensory neurons marked with green fluorescent protein (Boschat et al., 2002). More recently, activation of tomato-labeled V1rj2 or V1rj3 expressing vomeronasal cells by sulfated steroids has been shown (Haga-Yamanaka et al., 2014; Haga-Yamanaka et al., 2015). V1rj2 expressing cells responded to sulfated and glucuronidated estrogens, sulfated androgens, sulfated progesterones, and a corticosterone. V1rj3 expressing cells were more selective in their responses and reacted only to sulfated estrogens, androgens, and progesterone (Haga-Yamanaka et al., 2014; Haga-Yamanaka et al., 2015).

1.2.3.2 Vmn2r Expressing Vomeronasal Sensory Neurons

VSNs expressing Vmn2rs are resident in the basal VNO zone. These receptors are coexpressed with Gαo (Berghard and Buck, 1996; Jia and Halpern, 1996) and Gγ8 (Rünnenburger et al., 2002). In mouse, 122 of a total of 282 Vmn2r genes are intact genes (Martini et al., 2001; Young and Trask, 2007). Vmn2r expressing VSNs can be further subdivided into VSNs coexpressing a Vmn2r with an H2-Mv gene (Vmn2r+ H2-Mv) and VSNs expressing a Vmn2r without a H2-Mv gene (Vmn2r+ H2-Mv−). H2-Mv genes encode nine class I major histocompatibility complex (MHC) molecules. The associated MHC molecule is assumed to act as a co-factor, a chaperone, or a co-receptor for the given Vmn2r (Ishii et al., 2003; Loconto et al., 2003; Ishii and Mombaerts, 2008). A recent study deleted a 530 kb cluster of H2-Mv genes and showed lower sensitivity for a subset of VSNs lacking this cluster (Leinders-Zufall et al., 2014).

Vmn2r expressing cells have been shown to detect class I MHC-binding peptides (antigens) that alter female reproductive function (Leinders-Zufall et al., 2004; Leinders-Zufall et al., 2009; Boehm and Zufall, 2006; Leinders-Zufall et al., 2014). Related to this, pregnancy failure in freshly mated female mice (Bruce effect) could be evoked by the introduction of class I MHC-binding peptides of non-familiar males into familiar male urine that was given as stimulus (Leinders-Zufall et al., 2004). It has been shown that Vmn2r26 (V2r1b) expressing VSNs can be activated by antigens, such as SYFPEITHI, SEIDLILGY, and AYKDNRETI (Leinders-Zufall et al., 2004). Distinct derivatives of these substances also activated the examined cells. Moreover, Vmn2r81 (V2rf2) expressing cells reacted to the antigens SEIDLILGY and f-MFFINTLTL (Leinders-Zufall et al., 2014). These
findings provide support for an evolutionary link between recognition mechanisms in immune cells and subsets of VSNs.

Vmn2rs of the V2rp subfamily respond to exocrine gland-secreting peptides from tear fluid and saliva of mice (Kimoto et al., 2005; Dey and Matsunami, 2011). These peptides form a family of 38 related peptides of which 15 have been shown to elicit electrical responses in the VNO (Kimoto et al., 2007). Vmn2r116 (V2Rp5) is the best studied receptor detecting this substance class. VSNs that express Vmn2r116 are activated by exocrine gland-secreting peptide 1 (ESP1), a 7-kDa peptide secreted by the extraorbital lacrymal gland of male mice that enhances lordosis behavior in female mice. Knocking out the receptor gene abolishes VSN responses to ESP1 (Kimoto et al., 2005; Haga et al., 2010). Moreover, a single report described the activation of Vmn2r111 (V2rp2) by ESP5 and Vmn2r112 (V2rp1) by ESP5 and ESP6 (Dey and Matsunami, 2011). Recently, ESP22 that is present in tears of prepubertal mice was found to inhibit mating behavior of adult males (Ferrero et al., 2013).

Another potent ligand group assigned to the basal VNO layer is composed of major urinary proteins (MUPs) (Sturm et al., 2013). These proteins are proposed to operate as olfactory signals for conspecific recognition, due to their high polymorphism rate and the fact that they are genetically encoded (Cheetham et al., 2007). MUPs may elicit inbreeding avoidance, countermarking behavior, and female sexual attraction (Hurst et al., 2001; Sherborne et al., 2007; Roberts et al., 2010; Kaur et al., 2014). Additional to this already wide recognition capacity, they also play an important role in mediating aggressive behavior, conditioned learned spatial preference, and the detection of predators (Chamero et al., 2007, Chamero et al., 2011; Papes et al., 2010; Roberts et al., 2012). To date, no specific Vmn2r could be assigned to this ligand class thus far.

1.2.3.3 Formyl Peptide Receptor Expressing Vomeronasal Sensory Neurons

Other important G protein-coupled receptors expressed in VSNs are Fprs. Mice encode seven Fpr genes (Gao et al., 1998; Ye et al., 2009). Two independent studies recently discovered five murine Fprs in subpopulations of VSNs (Liberles et al., 2009; Rivière et al., 2009). Four Fprs (Fpr-rs3, Fpr-rs4, Fpr-rs6, and Fpr-rs7) are located in the apical layer, where they coexpress with Ga2. Fpr3 (formerly known as Fpr-rs1; see MGI gene ID: 1194495) is the only Fpr expressed by basal VSNs and coexpresses with Ga (Liberles et al., 2009; Rivière et al., 2009). Vomeronasal Fprs are not coexpressed with other types of vomeronasal receptors. Substances that are classified as Fpr agonists, such as the artificial W-peptide or the archetypical N-formylated peptide f-MLF, were shown to activate
vomeronasal Fprs (Rivière et al., 2009; Bufe et al., 2012; Bufe et al., 2015). However, their precise function is unknown, but some evidence suggests members of the Fpr family, particularly Fpr3, as current prime candidates for mediating vomeronasal pathogen sensing.

Figure 3. Receptors expressed in the vomeronasal organ. Schematic depiction of different receptor types expressed in murine VSNs. The number of known mouse genes is indicated below each structure. In the apical layer, 191 intact Vmn1r and four Fprs are expressed. In the basal layer, 122 intact Vmn2rs and Fpr3 are expressed. The receptors are expressed in non-overlapping subpopulations of VSNs. Figure was adapted and modified from Chamero et al., 2012.

1.3 Formyl Peptide Receptors

1.3.1 General Function of Formyl Peptide Receptors

Fprs are chemotactic G protein-coupled receptors (Bupe and Zufall, 2016; Dahlgren et al., 2016). They comprise a single 350 to 370 amino acid long polypeptide chain that forms seven transmembrane domains (Boulay et al., 1990). The transmembrane segments of Fpr genes are more conserved than their extracellular domains, which display significant variability (Migeotte et al., 2006; Fu et al., 2006).

Little is known about Fpr function in the olfactory system. However, their function in the immune system is intensely studied. There they are involved in chemoattraction of phagocytic immune cells and contribute to the recruitment of cells from the bone marrow to the blood stream and from the blood stream to inflammatory sites (Ye and Boulay, 1997). They can detect a large spectrum of chemical attractants either secreted by invading pathogens or released during inflammatory processes (Migeotte et al., 2006, Ye et al., 2009). Upon activation, Fprs mediate a variety of host defense mechanisms against invading microorganisms (Fu et al., 2006; Migeotte et al., 2006; Ye et al., 2009; Bloes et al., 2015). Besides their prominent role in chemotaxis, Fprs are also involved in the mobilization of
adhesion molecules from intracellular storage granules, the secretion of proteolytic enzymes and in reactive oxygen species production (Holland, 2013; Parker and Winterbourn, 2013).

To fulfill this plethora of functions Fprs are very broadly tuned and promiscuous receptors that are capable of sensing many different ligands of different substance classes. Their functional promiscuity becomes clear when looking at the wide range of recognized substances. These include inflammatory markers, such as LL-37 (Yang et al., 2000) and Annexin A1 (Ernst et al., 2004), viral peptides, such as several viral HIV-1 envelope proteins (Su et al., 1999a; Su et al., 1999b; Le et al., 2000), and host-derived peptides including mitochondrial peptides, such as different NADH-ubiquinone oxidoreductase chain, internal NADH dehydrogenase, and Cytochrome c oxidase subunit peptides (Rabiet et al., 2005; Gripentrog and Miettinen, 2008; Bufe et al., 2012). Furthermore, Fprs detect peptides associated to Alzheimer’s disease, such as serum amyloid A (Ye et al., 2009), Aβ42 (Le et al., 2001a; Tiffany et al., 2001), and humanin that protects cells from damage caused by Aβ42 (Harada et al., 2004), as well as some host-derived non-peptide agonists. Moreover, they can detect a variety of synthetic peptides, such as the hexa-peptides W-peptide or M-peptide (Fu et al., 2006; Ye et al., 2009; Bae et al., 2012; Bufe et al., 2012; He et al., 2013; Bufe et al., 2015; Bylund et al., 2014; Bufe and Zufall, 2016).

Currently best studied are bacterial peptide agonists with N-formylated peptides leading the way. Most known formylated peptides are derived from bacteria. f-MLF, derived from Escherichia coli, was one of the first characterized chemotactic peptides and has been studied extensively since its initial discovery in 1975 (Schiffmann et al., 1975). f-MLF is the smallest formyl peptide that displays full agonistic activity for human FPR1 and FPR2. Other bacterial peptides, such as peptides derived from Listeria monocytogenes (Rabiet et al., 2005; He et al., 2013) and phenol-soluble modulin peptide toxins derived from Staphylococcus aureus (Wang et al., 2007; Bloes et al., 2015), have been also identified as Fpr agonists. The detection mechanism of Fprs relies on specific peptide motifs that are present in sensed peptides (Bufe et al., 2012). Intriguingly, the motifs found in many formerly identified bacterial peptides are prime components of bacterial signal peptides (Bennet et al., 1980; Bufe and Zufall, 2016). In line with these findings, recent systematic structure-function studies of Fprs from six mammalian species revealed clear evidence that Fprs primarily focus on the detection of formylated signal peptides from bacteria. This is due to specific evolutionarily conserved pathogen associated molecular patterns that possibly allow for detection of more than 100,000 distinct formyl peptides (Bufe et al., 2015).
INTRODUCTION

Signal peptides are the first natural agonists identified for murine Fpr3. They guide newly synthesized proteins to the membrane transport machinery of a bacterium. Besides their differing amino acid sequences they share a conserved secondary structure that is largely \( \alpha \)-helical (von Heinje, 1985). They exhibit a typical motif combining a high degree of sequence flexibility and a conserved three-dimensional topology. Bacterial signal peptides comprise three typical domains: a 3-6 amino acid long N-terminal region (blue) starting with a methionine (M), an \( \alpha \)-helical hydrophobic h-region (green), and a C-terminal region (red), that contains a conserved signal peptidase (SPase) recognition motif (von Heinje, 1985; Dalbey et al., 2012). Figure was adapted from Bufe et al., 2015.

1.3.2 Genetics of Formyl Peptide Receptors

Fprs can be found in mammals (Liberles et al., 2009) and in other phylogenetic classes, such as birds and fish (Panaro et al., 2007; Bufe and Zufall, 2016). The human genome encodes three Fpr genes, FPRI, FPR2, and FPR3, in a cluster located on chromosomal region 19q13.3 (Figure 5B) (Bao et al., 1992; Alvarez et al., 1994). All three human Fpr genes are single copy genes with intron-less open reading frames, but they encode introns in their untranslated regions (Perez et al., 1992; Ye et al., 1992; Bao et al., 1992; Murphy et al., 1992).

Figure 5. Relationship and genomic organization of human and murine Fpr genes. A, dendrogram depicting amino acid sequence similarities between human and murine Fprs. B, organization of the three human Fpr genes located on chromosome 19q13.3. White and red boxes indicate non-coding or coding exonic sequence segments, respectively. C, organization of the nine functional and non-functional Fpr genes of the mouse located on chromosome 17A3.2. Red, purple, yellow, and white arrows represent presumably functional Fpr genes, pseudogenes, vomeronasal receptor genes, or other genes, respectively. hFPR = human Fpr; mFpr = murine Fpr. Figure was adapted from Migeotte et al., 2006.

Looking at the organization of Fpr genes in other species than human, the complex history and evolution of the receptor family becomes clear. In rodents, the Fpr gene cluster has undergone species-specific expansion (Gao et al., 1998; Wang and Ye, 2002). Thus, the mouse genome encodes seven full-length Fpr genes – Fpr1, Fpr2, Fpr3, Fpr-rs3, Fpr-rs4, Fpr-rs6, and Fpr-rs7 – and two pseudogenes \( \psi \)Fpr-rs2 (Fpr-rs8) and \( \psi \)Fpr-rs3 (Fpr-rs5) with
premature stop codons on chromosome 17A3.2 (Figure 5C) (Gao et al., 1998; Migeotte et al., 2006). ψFpr-rs2 has an open reading frame and its expression could be induced by LPS stimulation in spleen and bone marrow, which are both leukocyte accumulating organs (Tiffany et al., 2011). This suggests that the protein product of ψFpr-rs2 may fulfill a biological function.

1.3.3 Tissue Distribution of Formyl Peptide Receptors

Fpr expression is described in a variety of leukocytes. Human FPR1 has been described in tissue-residing neutrophil granulocytes, macrophages, microglia, dendritic cells, monocytes, and lymphocytes (Durstin et al., 1994; Lacy et al., 1995; Migeotte et al., 2005; Migeotte et al., 2006). Expression of human FPR1 was also reported for multiple non-immune organs and tissues including epithelial cells in organs with secretory functions, thyroid and cortical cells of the adrenal gland, liver, smooth muscle, brain, spinal cord, and both motor and sensory neurons (Becker et al., 1998; Ye et al., 2009). Human FPR2 expression has been reported for neutrophil granulocytes, monocytes, macrophages, T- and B-lymphocytes, microglial cells, platelets, hepatocytes, epithelial cells, microvascular endothelial cells, and fibroblasts (Le et al., 2001b; VanCompernolle et al., 2003; Czapiga et al., 2005; Migeotte et al., 2006). Expression of human FPR3 is less studied. This receptor has been found in fewer cell types than the other human Fprs (Ye et al., 2009). mRNA of the receptor has been detected in monocytes and dendritic cells, where it is thought to be the dominant formyl peptide receptor (Yang et al., 2001; Yang et al., 2002; Migeotte et al., 2005). RNA of human FPR3 has also been detected in a wide variety of tissues including lung, spleen, lymph nodes, trachea, placenta, liver, adrenal gland, small intestine, and in some other tissues at minor levels, by using quantitative reverse transcription polymerase chain reaction (RT-PCR) (Harada et al., 2004; Migeotte et al., 2006).

Expression of murine Fprs is not studied to the extent of human Fprs. Murine Fpr1 is expressed in dendritic cells, neutrophil granulocytes, and bone marrow cells (Gao et al., 1998; Lee et al., 2004; Southgate et al., 2008; Chiu et al., 2013). Moreover, transcripts for murine Fpr1 have been reported for spleen, lung, kidney, liver, the trigeminal nerve, and bone marrow cells (Migeotte et al., 2006; Chiu et al., 2013). Murine Fpr2 has been found in the same cells and organs as Fpr1, except for liver (Gao et al., 1998; Lee et al., 2004; Migeotte et al., 2006; Southgate et al., 2008; Chiu et al., 2013).

Investigations on murine Fpr3 expression are more ambiguous. One report described Fpr3 expression in the leukocyte accumulating organs spleen and lung, as well as in
Peripheral blood-derived leukocytes by northern blot analyses (Gao et al., 1998). However, the performed experiments lacked a cellular resolution and technical issues, such as cross-hybridization with other Fprs, could not be completely excluded. Moreover, several other studies could not confirm the finding of murine Fpr3 expression in leukocytes (Lee et al., 2004; Southgate et al., 2008; Rivière et al., 2009; Chiu et al., 2013).

The expression of the Fpr-rs genes is even less understood. Besides Fpr3, all of them are expressed in the VNO (Liberles et al., 2009; Rivière et al., 2009). Small amounts of Fpr-rs3 RNA were also found in skeletal muscle by northern blot analyses (Gao et al., 1998). Fpr-rs4 expression outside the olfactory system has not been reported yet. Fpr-rs6 RNA has been detected in brain, spleen, testis, and skeletal muscle. Fpr-rs7 expression has been described in heart, liver, lung, spleen, smooth muscle, pancreas by RT-PCR (Wang and Ye, 2002). Moreover, transcripts for Fpr-rs6 and Fpr-rs7 were identified from bone marrow-derived dendritic cells (Lee et al., 2004).

1.3.4 Relationship between Murine and Human Formyl Peptide Receptors

The evolutionary relationship between murine Fprs and their human counterparts are not understood in their entirety. In agreement with several publications, the mouse genes Fpr1 and Fpr2 are orthologs of human FPR1 and FPR2, respectively (Migeotte et al., 2006; Önnheim et al., 2008; Dahlgren et al., 2016). Functionally, murine and human Fpr1 and Fpr2 are very similar. Receptors of both species are very promiscuous (see chapter 1.3.1). Thus, it is not surprising that many ligands activating human Fprs also activate their mouse orthologs.

The relationship between murine Fpr3 and the human Fpr genes is insufficiently examined. Its sequence is most similar with that of human FPR2 with a significant overlap to human FPR3 (Hartt et al., 1999; Ye et al., 2009). The high sequence similarity of murine Fpr3 to these human immune receptors provides evidence for a possible expression of murine Fpr3 in the immune system. Furthermore, its orientation and position on the chromosome resembles that of human FPR3 (Figure 5B, C). Murine Fpr3 has only recently been deorphanized (Bufe et al., 2012; He et al., 2013; Bufe et al., 2015). Functionally, murine Fpr3 seems to share a high similarity with human FPR3 since both receptors are less promiscuous than Fpr1 and Fpr2 of both species. Calcium imaging experiments showed responses for murine and human Fpr1 and Fpr2 to a wide panel of bacterial signal peptides whereas Fpr3 of both species was much more narrowly tuned. These data provide first evidence for a functional orthology between murine and human Fpr3. However, to substantiate the definite relationship of murine Fpr3 to a possible human counterpart, further
functional investigations must be done. Fpr-rs3, Fpr-rs4, Fpr-rs6, and Fpr-rs7 seem to have no direct counterparts in the human genome (Figure 5A) (Migeotte et al., 2006).

1.4 Aims of this Work

Recently, formyl peptide receptors have been discovered in the mouse vomeronasal organ (Liberles et al., 2009; Rivière et al., 2009). However, the biological role of these vomeronasal Fprs is poorly understood but they are candidate pathogen sensors based on the function of structurally related Fprs in immune cells (Liberles et al., 2009; Rivière et al., 2009). Murine Fpr3 was the first vomeronasal Fpr that was recently deorphanized (Bufe et al., 2012). Furthermore, several studies have reported functional similarities between murine Fpr3 and the immune receptor human FPR3. Both receptors sense peptides of bacterial origin (Harada et al., 2004; Ernst et al., 2004; Bufe et al., 2015). However, differences in their expression patterns argue against a similar function for the two receptors. Murine Fpr3 is expressed in the vomeronasal organ, but reports about its presence in immune cells are controversial (Gao et al., 1998; Lee et al., 2004; Southgate et al., 2008; Rivière et al., 2009; Chiu et al., 2013). Human FPR3 is found in immune cells but adult humans lack a functional vomeronasal organ (Yang et al., 2001; Yang et al., 2002; Migeotte et al., 2005; Liman and Innan, 2003; Zhang and Webb, 2003). Thus, it is questionable if both receptors fulfill similar functions. Three main questions were proposed to understand the biological relationship between murine and human Fpr3:

1. What is the evolutionary relationship between murine and human Fpr3?
2. What is the functional relationship between murine and human Fpr3?
3. What is the precise expression pattern of murine Fpr3 in different tissues?

Careful characterization of murine Fpr3 expression with two newly generated antibodies showed that the receptor is expressed in the vomeronasal organ and immune system. Functional studies using heterologous calcium imaging provided clear evidence for similarities in the agonist responses of murine and human Fpr3.

Additionally, a large panel of natural Fpr3 knockout mouse strains that lack expression and function of this receptor are presented.
2 EXPERIMENTAL PROCEDURES

2.1 Ligands

To achieve the main goals of this work, it was relevant to gain insight into the function of murine and human Fpr3. Therefore, many different ligands of different origins and with different properties were used for functional experiments.

2.1.1 Synthetic Ligands

A major ligand group used in this study consisted of synthetic peptides, comprising W- and M-peptide in D- and L-conformation, and various W-peptide derivatives. Table 1 lists all synthetic peptide ligands used in this study with their primary structure, the company they were purchased from, their purity, the solvent they were diluted in, and the FL-number (FL#), which is the internal laboratory identifier.

Table 1. Synthetic ligands used in this study

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Primary Structure</th>
<th>Company</th>
<th>Purity (%)</th>
<th>Solvent</th>
<th>FL#</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-Peptide</td>
<td>KYYM\textsubscript{m}–NH\textsubscript{2}</td>
<td>Innovagen</td>
<td>&gt;95.00</td>
<td>C1</td>
<td>FL21</td>
</tr>
<tr>
<td>L-W-Peptide</td>
<td>KYYM\textsubscript{M}–NH\textsubscript{2}</td>
<td>Tocris</td>
<td>&gt;99.20</td>
<td>C1</td>
<td>FL22</td>
</tr>
<tr>
<td>D-M-Peptide</td>
<td>MKYAM\textsubscript{m}–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;99.60</td>
<td>C1</td>
<td>FL57</td>
</tr>
<tr>
<td>L-M-Peptide</td>
<td>MKYAM\textsubscript{M}–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;96.80</td>
<td>C1</td>
<td>FL56</td>
</tr>
<tr>
<td>W-Library Peptide 13b</td>
<td>KYYM[C–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;97.20</td>
<td>C1</td>
<td>FL46</td>
</tr>
<tr>
<td>W-Library Peptide 14</td>
<td>KYYM[a–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;98.50</td>
<td>C1</td>
<td>FL47</td>
</tr>
<tr>
<td>W-Library Peptide 29</td>
<td>KYYM[1–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;97.30</td>
<td>C1</td>
<td>FL81</td>
</tr>
<tr>
<td>W-Library Peptide 30</td>
<td>KYYM[0–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;99.90</td>
<td>C1</td>
<td>FL82</td>
</tr>
<tr>
<td>W-Library Peptide 28</td>
<td>KYYM[E–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;98.60</td>
<td>C1</td>
<td>FL80</td>
</tr>
<tr>
<td>W-Library Peptide 13</td>
<td>KYYM[c–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;95.00</td>
<td>C1</td>
<td>FL32</td>
</tr>
<tr>
<td>W-Library Peptide 33</td>
<td>W[YMV[hcy]–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;95.20</td>
<td>C1</td>
<td>FL85</td>
</tr>
<tr>
<td>W-Library Peptide 45</td>
<td>KYYM[q–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;97.70</td>
<td>C1</td>
<td>FL106</td>
</tr>
<tr>
<td>W-Library Peptide 27</td>
<td>KYYM[k–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;99.70</td>
<td>C1</td>
<td>FL79</td>
</tr>
<tr>
<td>W-Library Peptide 46</td>
<td>KYYM[e–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;97.50</td>
<td>C1</td>
<td>FL107</td>
</tr>
<tr>
<td>W-Library Peptide 92</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;95.19</td>
<td>C1</td>
<td>FL177</td>
</tr>
<tr>
<td>W-Library Peptide 93</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;98.11</td>
<td>C1</td>
<td>FL178</td>
</tr>
<tr>
<td>W-Library Peptide 94</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;98.52</td>
<td>C1</td>
<td>FL179</td>
</tr>
<tr>
<td>W-Library Peptide 91</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;97.45</td>
<td>C1</td>
<td>FL176</td>
</tr>
<tr>
<td>W-Library Peptide 87</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;95.26</td>
<td>C1</td>
<td>FL172</td>
</tr>
<tr>
<td>W-Library Peptide 88</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;95.31</td>
<td>C1</td>
<td>FL173</td>
</tr>
<tr>
<td>W-Library Peptide 89</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;95.75</td>
<td>C1</td>
<td>FL174</td>
</tr>
<tr>
<td>W-Library Peptide 90</td>
<td>KYYM[Am–NH\textsubscript{2}</td>
<td>VCPBIO</td>
<td>&gt;95.93</td>
<td>C1</td>
<td>FL175</td>
</tr>
<tr>
<td>W-Library Peptide 17</td>
<td>KAM[Am–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;99.00</td>
<td>C1</td>
<td>FL50</td>
</tr>
<tr>
<td>W-Library Peptide 16</td>
<td>KKM[Am–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;96.70</td>
<td>C1</td>
<td>FL49</td>
</tr>
<tr>
<td>W-Library Peptide 36</td>
<td>KKM[Am–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;96.70</td>
<td>C1</td>
<td>FL97</td>
</tr>
<tr>
<td>W-Library Peptide 34</td>
<td>KKM[Am–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;96.80</td>
<td>C1</td>
<td>FL95</td>
</tr>
<tr>
<td>W-Library Peptide 35</td>
<td>KKM[Am–NH\textsubscript{2}</td>
<td>GenScript Corporation</td>
<td>&gt;97.20</td>
<td>C1</td>
<td>FL96</td>
</tr>
</tbody>
</table>
2.1.2 Natural Ligands

Besides the synthetic ligands, various natural peptide ligands comprising murine, human, and bacterial peptides were used in this study. Table 2 displays all natural peptide ligands with their primary structure, their origin, the company they were acquired from, their purity, the solvent they were diluted in, and the FL-number (FL#), which is the internal laboratory identifier.

Table 2. Natural ligands used in this study

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Primary Structure</th>
<th>Origin</th>
<th>Company</th>
<th>Purity (%)</th>
<th>Solvent</th>
<th>FL#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine Peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac2-26</td>
<td>Ac-AMVSEFLKQAWFIENEQEVQTVK</td>
<td>Immune system</td>
<td>Tocris</td>
<td>&gt;95.00</td>
<td>C1</td>
<td>FL19</td>
</tr>
<tr>
<td>mATP6</td>
<td>f-MNENLF</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.44</td>
<td>C1</td>
<td>FL196</td>
</tr>
<tr>
<td>N-terminus of mND2</td>
<td>f-MNPITIL</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.33</td>
<td>C1</td>
<td>FL188</td>
</tr>
<tr>
<td>N-terminus of mND3</td>
<td>f-MNLYTV</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.52</td>
<td>C1</td>
<td>FL133</td>
</tr>
<tr>
<td>N-terminus of mND4</td>
<td>f-MLKILL</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.63</td>
<td>C1</td>
<td>FL189</td>
</tr>
<tr>
<td>N-terminus of mND4</td>
<td>f-MFSTFF</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.21</td>
<td>C1</td>
<td>FL190</td>
</tr>
<tr>
<td>N-terminus of mND5</td>
<td>f-MNIFTT</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.42</td>
<td>C1</td>
<td>FL191</td>
</tr>
<tr>
<td>N-terminus of mND6</td>
<td>f-MNNYIF</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;96.54</td>
<td>C1</td>
<td>FL192</td>
</tr>
<tr>
<td>N-terminus of mCyrb</td>
<td>f-MTNMRK</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.33</td>
<td>C1</td>
<td>FL195</td>
</tr>
<tr>
<td>N-terminus of mCOII</td>
<td>f-MTHQTH</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.74</td>
<td>C1</td>
<td>FL194</td>
</tr>
<tr>
<td>Human Peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-terminus of hND4</td>
<td>f-MLKLIV</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;96.96</td>
<td>C1</td>
<td>FL142</td>
</tr>
<tr>
<td>N-terminus of hND5</td>
<td>f-MTMHTT</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;98.00</td>
<td>C1</td>
<td>FL113</td>
</tr>
<tr>
<td>N-terminus of hND6</td>
<td>f-MNYALF</td>
<td>mitochondria</td>
<td>VCPBIO</td>
<td>&gt;95.97</td>
<td>C1</td>
<td>FL141</td>
</tr>
<tr>
<td>Bacterial Peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f-MIVILY</td>
<td>f-MIVILY</td>
<td>Listeria monocytophages</td>
<td>VCPBIO</td>
<td>&gt;96.34</td>
<td>C1</td>
<td>FL140</td>
</tr>
<tr>
<td>Streptococcus-SPI</td>
<td>f-MGFFFS</td>
<td>Streptococcus suis</td>
<td>VCPBIO</td>
<td>&gt;95.43</td>
<td>C1</td>
<td>FL134</td>
</tr>
<tr>
<td>Psychromonas-SPI</td>
<td>f-MLYFYS</td>
<td>Psychromonas ingrahami</td>
<td>VCPBIO</td>
<td>&gt;95.59</td>
<td>DMSO</td>
<td>FL207</td>
</tr>
<tr>
<td>Clostridium-SPI13</td>
<td>f-MKKNLV</td>
<td>Clostridium perfringens</td>
<td>United Biosystems</td>
<td>&gt;95.67</td>
<td>C1</td>
<td>FL239</td>
</tr>
<tr>
<td>Hydrogenobacter-SPI</td>
<td>f-MKKFLL</td>
<td>Hydrogenobacter thermophilus</td>
<td>United Biosystems</td>
<td>&gt;95.78</td>
<td>C1</td>
<td>FL245</td>
</tr>
<tr>
<td>Staphylococcus-SPI22</td>
<td>f-MKFKNI</td>
<td>Staphylococcus aureas</td>
<td>VCPBIO</td>
<td>&gt;95.30</td>
<td>C1</td>
<td>FL170</td>
</tr>
<tr>
<td>Salmonella-SPI24</td>
<td>f-MKFKFW</td>
<td>Salmonella enterica</td>
<td>VCPBIO</td>
<td>&gt;98.34</td>
<td>C1</td>
<td>FL185</td>
</tr>
<tr>
<td>Bacterial Signal Peptide Fragments (N-termini)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus-SPI22 FuLe</td>
<td>f-MKFKNILIALLLFFTSLVISPLNVKA</td>
<td>Staphylococcus aureas</td>
<td>VCPBIO</td>
<td>&gt;96.83</td>
<td>C1</td>
<td>FL180</td>
</tr>
</tbody>
</table>
EXPERIMENTAL PROCEDURES

2.2 Mouse Strains

In this thesis, experiments with a variety of different mouse strains were performed. For strain names, the nomenclature employed by the Mouse Genome Informatics database (ftp://ftp.informatics.jax.org/pub/reports/MGI_Strain.rpt) was used.

Table 3. Used mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Inbred Type</th>
<th>Source</th>
</tr>
</thead>
</table>
| C57Bl/6N | England    | Classical   | Trese Leinders-Zufall (Saarland University School of Medicine)  
|          |            |             | Dieter Bruns (Saarland University School of Medicine)           |
| NZB/Ola  | England    | Classical   | Reinhart Kluge (German Institute of Human Nutrition)            |
| 129X1/Sv | England    | Classical   | Trese Leinders-Zufall (Saarland University School of Medicine)  |
| BALB/cJ  | England    | Classical   | Trese Leinders-Zufall (Saarland University School of Medicine)  |
| FVB/Ncrl | Switzerland| Classical   | Frank Kirchhoff (Saarland University School of Medicine)        |
| SPRET/EiJ | Spain      | Wild-derived (spretus) |
| CAST/EiJ | Thailand   | Wild-derived (castaneus) |
| KAZ/DT  | Kazakhstan | Wild-derived (musculus) |
| CZE/DT  | Czech Republic | Wild-derived (musculus) |
| GER/DT  | Germany    | Wild-derived (domesticus) |
| FRA/DT  | France     | Wild-derived (domesticus) |

Mouse inbred strains can be classified into two groups: classical and wild-derived. While the genome of the classical inbred strains derived of only a few progenitors (Tucker et al., 1992; Beck et al., 2000) and comprises a mosaic of different mus musculus subspecies (Bonhomme et al., 1987), the genome of wild-derived inbred strains consists of wild living mice caught at different times and locations (Beck et al., 2000), and thus mostly represent the genome of the respective wild caught mus musculus subspecies.

2.3 Molecular Biology

2.3.1 Oligonucleotides

For this study, two types of primers were used – sequencing primers and polymerase chain reaction (PCR) primers. Sequencing primers were used in sequencing reactions to verify the nucleotide sequences of cloned genes. PCR primers were used for amplification of murine and human Fprs for cloning purposes. The housekeeping genes encoding glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and β-actin were also amplified with PCR primers as controls in PCR reactions. All primers were purchased from Sigma-Aldrich.
2.3.1.1 Sequencing Primers

Internal sequencing primers bind inside of a gene. Additionally, sequencing primers targeting the cytomegalovirus (CMV) promoter before the start codon (ATG) and the bovine growth hormone (BGH) polyadenylation site behind the stop codon (TGA) were used to ensure covering the complete amplified gene in order to obtain comprehensive analyses. Sequencing primers with their binding targets, their sequences and their PH-numbers (PH#), which are the internal laboratory identifiers are listed in table 4.

<table>
<thead>
<tr>
<th>Binding Target</th>
<th>Sequencing Primer Sequences [5' &gt; 3']</th>
<th>PH#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine Fpr3</td>
<td>Forward: GCTAGAAATGTTGTGGGTGCC</td>
<td>PH473</td>
</tr>
<tr>
<td></td>
<td>Forward: GATCAGATACTCGAGTTCACTAC</td>
<td>PH692</td>
</tr>
<tr>
<td></td>
<td>Forward: CTGAGATGCAGCACTGACTC</td>
<td>PH693</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGAAGTGAAGCCTAGCTG</td>
<td>PH694</td>
</tr>
<tr>
<td></td>
<td>Reverse: TAGAAGCAGACTGAGCAGG</td>
<td>PH695</td>
</tr>
<tr>
<td>CMV promoter</td>
<td>Forward: CGCAAATGCGGTAGCGAGCGTG</td>
<td>PH1</td>
</tr>
<tr>
<td>BGH polyadenylation site</td>
<td>Reverse: TAGAAGCAGACTGAGCAGG</td>
<td>PH2</td>
</tr>
</tbody>
</table>

2.3.1.2 PCR Primers

Primers for amplification of complete coding regions were designed to cover the start (ATG) and the stop codon (TGA). Forward primers comprised the start codon, whereas reverse primers included the stop codon. Primers that were used for gene amplification in PCR experiments, their targeted genes, and accession numbers are listed in table 5.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Numbers*</th>
<th>Synonyms*</th>
<th>Gene-Specific Primer Sequences [5' &gt; 3']</th>
<th>PH#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine Fpr3</td>
<td>NM_008042.2</td>
<td>ALX; Fpr-rs1;</td>
<td>Forward: ATGAAACCACTACTCTCCTCTTTGAT</td>
<td>PH354</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fpr-rs1; FprII;</td>
<td>Reverse: TATGCGTTTATATGCACTGAGA</td>
<td>PH355</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LXA4-R; Lxa4r</td>
<td>RNA Probe Primers for In Situ Hybridization:</td>
<td></td>
</tr>
<tr>
<td>Human FPR1</td>
<td>NM_001193306.1</td>
<td>FMLP; FPR</td>
<td>Forward: CACTACAAAGATTCACAAAAAGCCTTG</td>
<td>PH585</td>
</tr>
<tr>
<td>Human FPR2</td>
<td>NM_001462.3</td>
<td>ALX; FMLP-R2;</td>
<td>Reverse: CATTGCGCTGAACTCACTG</td>
<td>PH559</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FMLPX; FPR2A;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FPRH1; FPRH2;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPM1; HM63; LXA4R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human FPR3</td>
<td>NM_002030.4</td>
<td>FMLP-R2; HUMAN;</td>
<td>Forward: ATGAAACCACTACTCTCCTCTCT</td>
<td>PH466</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FMLP-R2; HUMAN</td>
<td>Reverse: CATTGCCGTGAACTCACTG</td>
<td>PH465</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_007393.5</td>
<td>Actx; beta-actin;</td>
<td>Forward: CTGGAAGCAGCTGGAAGGTGCA</td>
<td>PH467</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E430023M04Rik</td>
<td>Reverse: AAGGACTCTCCTGGAACATGCA</td>
<td>PH468</td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_001289726.1</td>
<td>Gapd</td>
<td>Forward: ACCACAGTCCATGGCATCAC</td>
<td>PH1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse: TCCACACAGCTGCGATGTA</td>
<td>PH2</td>
</tr>
</tbody>
</table>

* = National Center for Biotechnology Information (NCBI) data.
For cloning, PCR primers were attached to a sequence with several restriction sites to ensure directed insertion into expression vectors. To forward primers a *Hind*III- (AAGCTT), a *Sbf*I-site (CCTGCAGG), and a KOZAK sequence (GCCACC) was added upstream of the start codon (ATG) at the 5’ end.

- **forward primers:** 5’–\textcolor{blue}{AAGCTT} \textcolor{red}{CCTGCAGG} \textcolor{orange}{GCCACC} \textcolor{yellow}{ATG} – 3’
  \[\text{\textcolor{blue}{Hind}III-site}\]
  \[\text{\textcolor{red}{Sbf}I-site}\]
  \[\text{\textcolor{orange}{KOZAK} sequence}\]
  \[\text{\textcolor{yellow}{start codon}}\]

To reverse primers a stop codon (TGA), a *Sac*I- (GAGCTC), and a *Not*I-site (GCGGCCGC) was added downstream of the stop codon (ATG) at the 3’ end.

- **reverse primers:** 3’–\textcolor{red}{GCGGCCGC} \textcolor{blue}{GAGCTC} \textcolor{yellow}{TGA} – 5’
  \[\text{\textcolor{red}{Not}I-site}\]
  \[\text{\textcolor{blue}{Sac}I-site}\]
  \[\text{\textcolor{yellow}{stop codon}}\]

### 2.3.2 Polymerase Chain Reaction

PCR was performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) according to the manufacturer’s protocol. For each reaction, 0.5 µl cDNA and 10 pM of a forward and reverse primer were added to 10 µl 2× Phusion Master Mix, respectively, and filled up to a total volume of 20 µl with de-ionized water. The whole reaction was pipetted and mixed on ice. Reaction conditions for all primers were 98°C for 15 s followed by 35 cycles (denaturation: 98°C for 10 s, annealing: 64°C for 10 s, elongation: 72°C for 40 s), and 72°C for 20 s. Subsequently, PCR products were purified.

### 2.3.3 PCR Templates

PCR templates for this study were obtained via RNA isolation (see chapter 2.3.4), subsequent cDNA synthesis (see chapter 2.3.5), and by direct extraction of genomic DNA (see chapter 2.3.6). Templates were collected from different mouse strains and different
tissues. Table 6 outlines all used PCR templates, the organs and mouse strains they were gathered from, and the providers of the used mice.

<table>
<thead>
<tr>
<th>Template</th>
<th>Organ</th>
<th>Mouse Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>Blood</td>
<td>C57</td>
<td>Purchased from Zyagen</td>
</tr>
<tr>
<td>cDNA</td>
<td>VNO Bone Marrow Cells</td>
<td>C57Bl/6NCrl</td>
<td>Trese Leinders-Zufall and Dieter Bruns (Saarland University School of Medicine)</td>
</tr>
<tr>
<td></td>
<td>VNO</td>
<td>NZB/Ola</td>
<td>Reinhart Kluge (German Institute of Human Nutrition)</td>
</tr>
<tr>
<td></td>
<td>VNO</td>
<td>129X1/Sv</td>
<td>Trese Leinders-Zufall (Saarland University School of Medicine)</td>
</tr>
<tr>
<td></td>
<td>VNO</td>
<td>BALB/cJ</td>
<td>Trese Leinders-Zufall (Saarland University School of Medicine)</td>
</tr>
<tr>
<td></td>
<td>VNO</td>
<td>FVB/NCrl</td>
<td>Frank Kirchhoff (Saarland University School of Medicine)</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>Ear</td>
<td>SPRET/EiJ</td>
<td>Diethard Tautz (Max-Planck-Institut für Evolutionsbiologie)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAST/EiJ</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>KAZ/DT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CZE/DT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GER/DT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FRA/DT</td>
<td></td>
</tr>
</tbody>
</table>

### 2.3.4 RNA Isolation

RNA from VNO and bone marrow of mice was obtained with the innuPREP RNA Mini Kit (Analytik Jena AG) according to the manufacturer’s protocol. For lysis, tissue was incubated in 400 µl RL buffer for 2 min at room temperature (RT) and subsequently fully resuspended. For removal of genomic DNA, the suspension was transferred to a Spin Filter D and centrifuged at 10,000× g for 2 min and 400 µl 70% (v/v) ethanol was added to the filtrate. Binding of RNA was achieved by transferring the sample to Spin Filter R and subsequent centrifugation at 10,000× g for 2 min. For on-column DNase I digestion, 300 µl Washing Solution HS were added to the filter and centrifuged at 10,000× g for 1 min. A DNase I/Digestion Buffer mix (40 Kunitz Units innuPREP DNase I in 80 µl DNase I Digestion Buffer) was applied to the filter and incubated for 15 min at RT. Subsequently, 300 µl Washing Buffer HS were added and centrifuged at 10,000× g for 1 min. For washing, 750 µl LS Buffer were added and centrifuged at 10,000× g for 1 min. Ethanol was removed by spinning the column again after discarding the flow-through at 10,000× g for 3 min. RNA was eluted with RNase-free water after incubation of 1 min at RT and subsequent centrifugation at 6,000× g for 1 min. Quality was assessed by gel electrophoresis and photometric measurements. After isolation, RNA was used to synthesize cDNA.
2.3.5 cDNA Synthesis

cDNA was synthesized from 0.5 µg total RNA using the Smart cDNA Synthesis protocol (Clontech) and Superscript II Reverse Transcriptase (Invitrogen). Therefore, 5 µl reverse transcriptase buffer (2µl 5× First Strand Buffer, 4 mM dichlorodiphenyltrichloroethane (DDT), 12.5 mM dNTP-Mix (3.125 mM of dATP, dTTP, dGTP, and dCTP), 40 Units MMLV reverse transcriptase RNase H⁻ (Gibco), 10 Units RiboLock RNase Inhibitor (Thermo Scientific), and 1.15 µl water treated with diethyl dicarbonate (DEPC)) were added to 0.1 µg to 1 µg RNA including 2 µM CDS Primer and 2 µM Smart II Primer. The whole reaction was pipetted and mixed on ice. Synthesis was achieved by increasing heating steps starting at 42°C for 30 min, followed by 45° for 10 min, 50°C for 10 min, 55°C for 10 min, and 65°C for 5 min. After synthesis, cDNA was diluted in 100 µl de-ionized water and subsequently used as template in PCR reactions.

2.3.6 Extraction of Genomic DNA

Genomic DNA was extracted from the VNO or ear stamps of mice with the Blood DNA Mini Kit (PEQLAB) according to the manufacturer’s protocol. For lysis 250 µl PBS, 25 µl OB-Protease, and 250 µl BL-Buffer were added to the tissue in a 1.5 ml Safe-lock tube (Eppendorf) and vortexed for 10 s. Afterwards, it was incubated at 70°C for 10 min. For binding of DNA, 260 µl isopropanol was added and mixed. Subsequently, the sample was transferred to the filter column and centrifuged at 8,000×g for 1 min. Flow-through was discarded. The column was washed twice with 600 µl washing buffer supplemented with ethanol. Flow-through was discarded. To remove ethanol, the column was centrifuged at 8,000×g for 2 min. Genomic DNA was eluted in 200 µl Elution-Buffer after 2 min via centrifugation at 5,000×g for 1 min and afterwards used as template in PCR reactions.

2.3.7 Purification of PCR Products

PCR products were purified with the MinElute PCR Purification Kit (Qiagen) according to the manufacturer’s protocol. Briefly, PCR products were diluted 1:5 with binding buffer, mixed via pipetting up and down and transferred on centrifugation columns. After centrifugation for 1 min at 14,000 rpm and RT the reactions were rinsed with 750 µl washing buffer. Subsequently, the reactions were centrifuged again, to eliminate alcohol remains. Purified DNA was eluted in 15 µl elution buffer via centrifugation for 1 min at 14,000 rpm and RT. Afterwards, purified PCR products were analyzed by separation with agarose gel electrophoresis.
2.3.8 Gel Electrophoresis

DNA was size separated in 1% (m/v) agarose gels. As electrophoresis buffer 0.5× TBE (de-ionized water containing 44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA-Na$_2$ (pH 8)) and ethidium bromide (Carl Roth®) was used. To obtain a gel agarose was added to the buffer. 10 µl DNA solution (1 µl in the case of a PCR sample) were adjusted to 12 µl with 6X Orange DNA Loading Dye (Thermo Scientific). Reference was 6 µl FastRuler™ Middle Range DNA Ladder (Thermo Scientific). The duration of an electrophoresis was 30 min under constant voltage of 80 V.

2.3.9 RNA Quantification

Quantification of RNA probes was performed in gel electrophoresis chambers pretreated with 0.3% (v/v) H$_2$O$_2$ for 24 h and RNase-free agarose was used. References, to determine RNA probe concentration, were 20 ng, 40 ng, 60 ng, 80 ng, and 100 ng of non-labeled probes that were loaded on the gel. Molecular weight standard was the RiboRuler™ High Range RNA Ladder (Thermo Scientific).

2.3.10 Enzymatic DNA Digestion

Subcloning of PCR products into vectors was performed by digesting purified PCR products and vectors with different restriction enzymes. PCR products were digested with SbfI and NotI. Digestions were performed after the Thermo Scientific™ FastDigest™ (Thermo Scientific) protocol. Therefore, 1 µl 10X FastDigest buffer, 0.5 µl of each used restriction enzyme, and ~200 ng purified PCR product or 1 µg plasmid vector were adjusted to 10 µl with de-ionized water. Additional 0.5 µl thermosensitive alkaline phosphatase (TSAP; Promega) were added to vector digestions. The reaction was incubated for 1 h at 37°C and could be afterwards used for ligation into expression vectors.

To identify bacterial clones carrying the desired receptor, restriction analyses were performed with the purified plasmids. Restriction analyses were performed with EcoRI and SacI. Digestion reactions were analyzed for specific band patterns on agarose gels. Restriction sites were predicted with the Vector NTI® software (life technologies).

2.3.11 Expression Vectors

PCR products containing the complete coding sequence of a gene were ligated into mammalian expression vectors in 5’ → 3’ orientation. For calcium imaging experiments, Fprs
were inserted into a slightly modified version of pcDNA™3.1 (Thermo Scientific) that contained a SbfI restriction site.

For immunocytochemical detection, Fprs were inserted into the pcDNA™5/FRT/TO vector (Thermo Scientific), a tetracycline inducible plasmid that can be used in mammalian cells with the Flp-In T-REx™ system (Thermo Scientific). It was also modified in its multiple cloning site (MCS) by adding an extra SbfI restriction site. In addition, the plasmid contains the sequence for the first 39 amino acids of bovine rhodopsin (Rho-tag). When the inserted gene is expressed, the Rho-tag is fused to the N-terminus of the receptor gene and can be detected by an anti-Rho antibody. This allows examination of protein expression for the inserted receptor via immunofluorescence analyses. Fpr genes were cloned into SbfI and NotI sites within the modified pcDNA™3.1 (Thermo Scientific) and pcDNA™5/FRT/TO vectors. Figure 6 shows the altered cloning sites of the two vectors.

\[ Fpr3\Delta_{424-435} \] was subcloned from the modified pcDNA™5/FRT/TO vector to an existing plasmid containing 11 amino acids derived from herpes simplex virus glycoprotein D (HSV-tag) downstream of its multiple cloning site. This subcloning was realized with HindIII and NotI and resulted in an Fpr3Δ_{424-435} receptor with an N-terminal Rho-tag and a C-terminal HSV-tag that was expressed as fusion protein. Thereby, the extent of the expression of Fpr3Δ_{424-435} in transiently transfected HEK293T cells could be examined in immunocytochemistry experiments targeting the Rho- and HSV-tag. Figure 7 shows the altered cloning site of the described expression vector. All plasmids carry an ampicillin resistance gene for selective growth in bacteria.
2.3.12 DNA Ligation

Ligation of digested and purified DNA (plasmids and PCR products) was performed with the Fast-Link™ DNA Ligation Kit (LK0750H; Epicentre Biotechnologies) according to the manufacturer’s protocol. Therefore, 0.5 µl 10× Fast-Link™ Ligation Buffer, 0.5 µl ATP, 0.5 µl Fast-Link™ DNA Ligase, 10 ng to 20 ng insert DNA, and 5 ng to 10 ng vector DNA were adjusted to 5 µl with de-ionized water. Reactions were incubated overnight at 4°C or 1 h at RT. Ligation products may be used for transformation of competent Escherichia coli.

2.3.13 Transformation of Competent Escherichia coli

Ligation products were transformed via heat-shock into competent Escherichia coli 10-β bacteria (competence > 10⁹ CFU; New England Biolabs). Stocks of Escherichia coli 10-β were stored at -80°C and thawed on ice. 1 µl ligation product was added to a 15 µl bacteria aliquot and incubated on ice for 10 min. Heat-shock was performed for 30 s at 42°C in the water bath. Afterwards, bacteria were held on ice for 1 min. 150 µl SOC-medium (2% (m/v) casein hydrolyzate, 5% (m/v) yeast extract, 0.05% (m/v) NaCl, 2.5 mM KCl, 10 mM MgCl₂, and 20 mM D-Glucose, adjusted to pH 7.4) were given to the bacteria for regeneration and shaken at 220 rpm and 37°C for 1 h. Subsequently, bacteria were spread on agar plates containing 50 µg/ml ampicillin and incubated overnight at 37°C.

2.3.14 Isolation of Plasmid DNA from Bacterial Cultures

At least three bacteria colonies were picked for each genetic construct for restriction analysis. Clones positive for the restriction analysis were inoculated in 5 ml of 2x YT-medium (1.6% (m/v) casein hydrolyzate, 1% (m/v) yeast extract, 0.5% (m/v) NaCl, adjusted to pH 7.4) containing 50 µg/ml ampicillin and kept shaking overnight at 37°C. Grown bacteria cultures were harvested by centrifugation for 10 min at 3,200×g and RT.

Figure 7. Schema of the expression cassette of pcDNA™5/FRT/TO for Fpr3Δ424-435. Fpr3Δ424-435 was subcloned into pcDNA5/FRT/TO carrying an upstream Rho-tag and downstream 11 amino acids of the herpes simplex virus glycoprotein D (HSV-tag). Start (ATG) and Stop (TGA) codons are indicated below. Restriction sites are shown as black bars. Corresponding restriction enzymes are shown above the gene cassettes. BGH = Bovine Growth Hormone polyadenylation site; CMV = cytomegalovirus promoter sequence; HSV = herpes simplex virus derived fusion sequence; Rho = rhodopsin-derived fusion sequence; TO = tetracycline operator.
Isolation of plasmid DNA from the bacteria was realized with the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer’s protocol.

2.3.15 Determining DNA and RNA Concentration

Concentration and purity of DNA and RNA solutions were determined with the Ultrospec 2100 pro photometer (Amersham Biosciences). Therefore, light absorption at 260 nm (A_{260}) and 280 nm (A_{280}) was measured. Solutions were diluted 1:25 and measured in a 10 mm path length quartz cuvette. Values were determined in comparison to blank reference. Only DNA solutions with an A_{260/280} quotient between 1.7 and 1.9, and RNA solutions with an A_{260/280} quotient between 1.8 and 2.0 were used in experiments.

2.3.16 DNA Sequencing

DNA sequencing of PCR amplificates was performed by the Seqlab Co. (Göttingen, Germany) according to their guidelines. Primers used for generation of PCR products were also used for sequencing (see chapter 2.3.1.2; Table 5). Newly produced constructs were also sequenced with primers against sites resident in the cloning vectors: the human cytomegalovirus promoter sequence (CMV) and the bovine growth hormone poly-adenylation site (BGH). Sequencing reactions typically spanned ~1,000 bp. To cover the whole length of a given sequence, genes >1,000 bp were also sequenced with internal sequencing primers (see chapter 2.3.1.1; Table 4).

2.3.17 Generation of Bacterial Glycerol Stocks

Glycerol stocks of bacteria were acquired from 1 ml of an overnight culture. It was centrifuged at 5,000 rpm for 3 min. After discarding the supernatant the bacteria pellet was resuspended in 1 ml LB-medium (1% (m/v) casein hydrolyzate, 0.5% (m/v) yeast extract, 1% (m/v) NaCl, adjusted to pH 7.4) with 15% (v/v) glycerol and stored at -80°C.

2.3.18 Formyl Peptide Receptor Genes

Murine Fpr1 corresponds to the NCBI Reference Sequence NM_013521.2 with exchanges of T879C and G408A. Murine Fpr2 resembles the sequence of NM_008039.2 with a C192T exchange. Murine Fpr3 isolated from vomeronasal cDNA corresponds perfectly to NM_008042.2. Fpr3Δ_{424-435} is identical to Fpr3 except for a 12 nucleotide in-frame deletion spanning base pairs 424 to 435. Fpr-rs3 and Fpr-rs6 are identical to NM_008040.2 and
NM_177316.2, respectively. Fpr-rs4 corresponds to NM_008041.2 but with A69T and G577A exchanges. Fpr-rs7 corresponds to AF437513 with T441G and T500C exchanges. These deviations were also observed in genomic DNA of C57BL/6J mice. Human FPR1 resembles to NM_001193306.1 with V111L, R163H, and N192K exchanges. Human FPR2 and human FPR3 correspond to NM_001462.3 and NM_002030.3, respectively. Sequences for the full coding regions of all used receptors have been annotated (Bufo et al., 2015).

2.4 HEK293T Cell Culture

2.4.1 HEK293T Cells

HEK293T PEAKrapid cells (ATCC®; CRL-11268™) were used to investigate Fprs in a heterologous cell system. HEK293T PEAKrapid cells constitutively express the simian virus 40 (SV40) large T antigen and thus support the replication of recombinant plasmids with the SV40 origin of replication. The plasmid vectors used in this study provide the SV40 origin of replication.

2.4.2 Cell Culture Media

Table 7. Cell culture media

<table>
<thead>
<tr>
<th>HEK293T Culture Medium</th>
<th>Cryopreservation Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic Medium</td>
<td>Dulbecco’s Modified Eagle Medium (DMEM; D6429; Sigma-Aldrich)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (Sigma-Aldrich)</td>
<td>1% (v/v) = 10,000 Units/ml Penicillin, 10 mg/ml Streptomycin</td>
</tr>
<tr>
<td>L-Glutamine (Sigma-Aldrich)</td>
<td>2 mM</td>
</tr>
<tr>
<td>FCS (Sigma-Aldrich)</td>
<td>10% (v/v) (heat inactivated for 20 min at 56°C in a water bath)</td>
</tr>
<tr>
<td>DMSO</td>
<td>/</td>
</tr>
<tr>
<td>Storage</td>
<td>4°C</td>
</tr>
</tbody>
</table>

2.4.3 Cultivating Culture Cells

Cells were grown in 75 cm² culture flasks in 20 ml of appropriate culture medium (see chapter 2.4.2) and kept until 80% to 90% confluency. Propagation was realized by rinsing with 10 ml of Dulbecco’s Phosphate Buffered Saline (Sigma-Aldrich). Afterwards, cells were incubated in 5 ml Trypsin-EDTA for 3 min at RT until they detached from the flask bottom. Digestion was stopped by adding 5 ml appropriate culture medium. Separation of cells was performed by pipetting carefully up and down. After centrifugation at 900×g for 3 min cells were resuspended gently in 10 ml fresh culture medium. 1 ml of the suspension
EXPERIMENTAL PROCEDURES

was used to inoculate a new 75 cm² flask. HEK293T PEAKrapid cells were propagated until 30 passages. Then a new cell stock aliquot was thawed (see chapter 2.4.5).

For heterologous calcium imaging and immunocytochemistry experiments, black 96 well µCLEAR-Plates (Greiner bio-one) were coated by incubation with 50 µl 10 µg/ml poly-D-lysine dissolved in PBS for 30 min at RT. Afterwards, cells were seeded at 20% to 30% confluency in each well and incubated for 24 h (37°C, 5% (v/v) CO₂).

2.4.4 Transient Transfection of Culture Cells

When reaching 50% to 70% confluency, cells were transfected with the poly cationic DNA transfection reagent JetPEI™ (PEQLAB) according to the manufacturer’s protocol. Per well (96-well plate) a total of 0.25 µg DNA were diluted in 10 µl of 150 mM NaCl (4°C). In another tube, 0.5 µl JetPEI™ were added to another 10 µl of 150 mM NaCl (4°C). DNA was added to JetPEI™, briefly mixed, and incubated for 30 min at RT. Afterwards, the total 20 µl were added to a well containing seeded cells in 100 µl HEK293T culture medium (see chapter 2.4.2). For immunocytochemical analyses 0.25 µg plasmid DNA encoding a receptor were transfected. For calcium imaging experiments 0.125 µg DNA plasmid encoding a receptor were cotransfected with equal amounts of a plasmid encoding the G protein α16 subunit (Gα16). The total amount of transfected DNA was kept constant. Cells were dye loaded and imaged 48 h after transfection, with cell density of approximately 50,000 cells/well.

2.4.5 Thawing of Cryopreserved Culture Cells

For inoculation of new cell passages, stored cells were briefly thawed at 37°C in the water bath and quickly transferred to a 10 cm² culture flask (Sarstedt) filled with 37°C warm HEK293T culture medium (see chapter 2.4.2). After 4 h, the medium was replaced with fresh medium to wash out remaining DMSO and cell debris. When reaching 80% confluency, cells were carefully detached from the flask bottom, transferred to a 75 cm² culture flask (Sarstedt) and cultivated in appropriate culture medium (see chapter 2.4.2).

2.4.6 Storage of Culture Cells

HEK293T PEAKrapid cells were stored in cryopreservation medium (see chapter 2.4.2) and frozen to -80°C at -1 K/min using a Nalgene® Mr. Frosty cryo container (Sigma-Aldrich). Afterwards, they were stored in liquid nitrogen at -196°C.
2.5 High-Throughput Calcium Imaging

2.5.1 Cell Population Calcium Imaging

2.5.1.1 Dye Loading of HEK293T Cells for Cell Population Calcium Imaging

For calcium imaging measurements of cell populations, transiently transfected HEK293T cells (see chapter 2.4.4) were loaded with the fluorescence dye Fluo-4, AM (Molecular Probes). 48 h after transfection culture medium of each well of a given 96-well plate was exchanged to loading buffer (50 µl C1 buffer (130 mM NaCl, 5 mM KCl, 10 mM Na-HEPES, 2 mM CaCl₂, and 10 mM D-Glucose, adjusted to pH 7.4 with NaOH) containing 0.07% (m/v) Probenecid (Sigma-Aldrich) and 2 µM Fluo-4, AM). Cells were incubated for 2 h at RT in the dark. Subsequently, they were washed three times with C1 buffer using the ELx50 ELISA cell-washer (BioTek) and measured.

2.5.1.2 Data Acquisition for Cell Population Calcium Imaging with the FLIPR

For heterologous high-throughput calcium imaging on cell populations the fluorometric imaging plate reader (FLIPR; Molecular Devices) was used. This device is equipped with an automated overhead pipetting unit that can apply fluids (e.g. ligands) to all wells of a 96-well plate simultaneously. It can detect fluorescence signals in all wells and allows the examination of up to 96 stimuli for different transfection conditions simultaneously. Because of this, the FLIPR is well-suited for ligand screening and the examination of concentration-dependent responses of receptors upon different stimuli. Excitation of prepared cells is achieved with a water-cooled argon laser at 488 nm. The fluorescence detection technology is based on a charge-coupled device (CCD) camera setup whose detection optics are optimized for signals from a cell monolayer at the bottom of the well. The overall fluorescence adds up to one single signal that constitutes the response of the whole cell population in the well. Fluorescence is measured from each well independently and converted into a numerical value.

2.5.1.3 Analysis of FLIPR Experiments

Cell population responses of transfected HEK293T cells (~50,000 cells/well) were recorded using the FLIPR system. Response amplitudes (ΔF/F₀) were calculated by dividing the maximal change in fluorescence after ligand application (ΔF = Fₘₐₓ – Fₘᵟᵢₙ) by baseline fluorescence (F₀) (Figure 8A). In all experiments, a buffer control was measured in separate
wells to determine the maximal baseline variations without stimulation. The mean amplitude of control wells determined in a given experiment was then subtracted from all mean amplitudes obtained upon stimuli. This procedure prevented baseline variations in negative wells from being interpreted as signal amplitudes. In FLIPR experiments, quantifications and resulting bar charts represent signal amplitudes. Concentration-response curves were performed applying decreasing concentrations of a ligand to different wells (Figure 8B) and calculated with Graph Pad Prism 5.0 (GraphPad Software) (see chapter 2.9.2) (Figure 8C). All experiments were performed at least in duplicate wells using at least three independent transfections.

Figure 8. Representative responses in FLIPR experiments. A, maximal change in fluorescence after ligand (left panel) or buffer (right panel) application. Shown is the fluorescence minimum (F_{min}) and the fluorescence maximum (F_{max}) in blue, and the baseline fluorescence (F_{0}) in red. The maximal change in fluorescence is represented by ΔF. Response amplitudes were calculated with the formula ΔF/F_{0}. B, representative individual signals of a concentration-response curve obtained with decreasing ligand-concentrations in individual wells. C, Concentration-response curve calculated with the values of three independent experiments. Scale bars, vertical 5,000 RFU; horizontal 100 s.

2.5.2 Single Cell Calcium Imaging

2.5.2.1 Dye Loading of HEK293T Cells for Single Cell Calcium Imaging

For single cell calcium imaging measurements, transiently transfected HEK293T cells (see chapter 2.4.4) were loaded with the ratiometric calcium sensitive dye Fura-2, AM (Molecular Probes). 48 h after transfection culture medium of each well of a given 96-well plate was exchanged with Fura-2 loading buffer (100 µl Cl buffer containing
5 mM Trypan Red Plus™ (AAT Bioquest) and 2 µM Fura-2, AM). Afterwards, cells were incubated for 2 h at RT in the dark and washed three times with C1 buffer using the ELx50 ELISA cell-washer (BioTek).

### 2.5.2.2 Data Acquisition for Single Cell Calcium Imaging with the Bioimager

Heterologous calcium imaging in single cell resolution was performed with the Bioimager BD Pathway 855 (Bioimager; BD Biosciences). This device is a fully automated confocal microscope with an automated pipetting unit. It allows high-throughput live cell analyses of living cells with optional confocal imaging. Automated sequences of liquid (e.g., ligand) applications on 96-well plates with simultaneous single cell resolution live imaging can be programmed. Because of its resolution, responses of single cells can be detected in a well. Therefore, the Bioimager is well-suited for examination of signals that would be overlooked in cell population measurements and for high-throughput immunocytochemistry experiments that require single cell resolution. 103 W mercury short arc lamps (Chroma) provide a broad excitation spectrum of 330 nm to 900 nm. A set of dichroitic mirrors, excitation and absorption filters enable various settings for excitation and absorption. Pictures are taken with a high resolution ORCA-ER CCD camera (Hamamatsu). The optical apparatus contains several Olympus objectives that deliver magnifications of 4× to 60×.

### 2.5.2.3 Analysis of Bioimager Experiments

For automated single cell calcium imaging of transfected HEK293T cells (~50,000 cells/well) the BD Pathway 855 Bioimaging system was used. Calcium-dependent ratiometric fluorescence signals were recorded at 0.5 Hz. 30 µM ATP were applied after the stimulus and served as a positive control to monitor cell viability. Prior to the stimulus, C1 buffer was applied to control for mechanical artifacts. Cells that responded to C1 buffer (negative control) or lacked response to ATP (positive control) were excluded from the analysis. Images were taken with the Bioimaging system and quantified using Attovision Software (BD Biosciences) and Excel2010. In quantifications of Bioimager experiments, bar heights represent the percentage of responding cells.
2.6 Immunocytochemistry

2.6.1 Preparation of Samples for Immunocytochemistry

2.6.1.1 Dissociation of Vomeronasal Tissue

VNO epithelium of 8-12 week old mice was detached from the cartilage and minced in PBS at 4°C (Chamero et al., 2011; Pérez-Gómez et al., 2015). Pooled tissue from three to five mice was incubated for 20 min at 37°C in 1 ml PBS supplemented with papain (0.22 U/ml; Worthington), 1.1 mM EDTA (Thermo Scientific), and 5.5 mM L-cysteine hydrochloride (Sigma-Aldrich). Subsequently, cells were kept on ice for 5 min in 1 ml DNase buffer (600 µl PBS with 400 µl 5× Colorless GoTaq® Reaction Buffer (Promega) and 50 U DNase I (Thermo Scientific)). Thereafter, the reaction was stopped by adding 10 ml DMEM (Invitrogen) supplemented with 10% (v/v) FCS, and centrifuged for 5 min at 1,000×g and 4°C. After removal of supernatant, cells were resuspended in 200 µl DMEM supplemented with 10% (v/v) FCS and gently extruded by pipetting. Supernatant containing dissociated cells was seeded on coverslips coated with concanavalin-A (0.5 mg/ml, overnight at 4°C; Sigma-Aldrich) and incubated for 1 h at 37°C and 5% (v/v) CO2.

2.6.1.2 Preparation of Blood Cells

Blood samples were obtained from 8-12 week old mice that were euthanized with CO2. Blood (10 µl) was deposited on a microscope slide (Superfrost Plus; Menzel-Gläser) and smeared immediately. After drying blood smears were ready for experiments.

2.6.1.3 Preparation of Bone Marrow Cells

Isolation of bone marrow cells was performed as described (Boxio et al., 2004). 8-12 week old mice were euthanized with CO2 and decapitated. Femoral bones were isolated and stored in ice-cold calcium/magnesium-free Hank’s balanced salt solution (HBSS) buffer (Gibco) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) for 10 min. Epiphysis was removed from both ends of the bone and 1-2 ml HBSS with HEPES were forced through the bone shaft with a syringe (needle: 20G) to flush out the bone marrow. Bone marrow suspension was counted with a MOXI Z cell counter (Orflo) using Type S Moxi Z Cassettes (Orflo) and centrifuged at 300×g (brake on lowest level) for 8 min at 4°C. Supernatant was discarded and the cell pellet was resuspended in Roswell Park Memorial Institute medium (RPMI-1640, Gibco) containing 100 U/ml penicillin,
EXPERIMENTAL PROCEDURES

0.1 mg/ml streptomycin, 5 mM L-glutamine, and 10% (v/v) FCS (at a concentration of 4x 10^6 cells/ml. Cells were seeded on petri dishes (~1x 10^7 cells/dish) and incubated for 2 h at 37°C and 5% (v/v) CO₂. Stimulation of bone marrow cells was achieved by adding 150 µg/ml lipopolysaccharide of Salmonella enteriditis (Sigma-Aldrich) into culture medium of seeded cells. Stimulated and unstimulated samples were incubated for additional 8 h at 37°C and 5% (v/v) CO₂ before use in experiments.

2.6.2 Immunostaining Protocol

Cells were fixed for 4 min in 4% (m/v) methanol-free paraformaldehyde (Polyscience), rinsed in PBS, and treated with a blocking solution (PBS supplemented with 5% (v/v) FCS) and 0.25% (v/v) Triton-X100 for 30 min at RT. Thereafter, cells were incubated overnight at 4°C with primary antibody in blocking solution. After rinsing with PBS, staining was obtained by sample incubation for 60 min at RT with fluorescence-conjugated secondary antibody in blocking solution containing Hoechst33342 (1 µg/ml; Hoechst) to counterstain cell nuclei. The same immunocytochemistry protocol was used for HEK293T cells, dissociated VNO cells, and leukocytes from blood and bone marrow.

2.6.2.1 Image Acquisition and Data Analysis for General Immunostainings

All representative images from vomeronasal, blood, and bone marrow cells were taken with an Olympus BX61 fluorescence microscope with an X-Cite® SERIES 120PC (EXFO) light source. Representative pictures of HEK293T cells and montage pictures for quantification were taken with the BD Pathway 855 Bioimaging system (BD Biosciences). Quantifications were evaluated with BD-image Explorer software (BD Biosciences).

2.6.3 Antibodies

2.6.3.1 Used Antibodies

Primary antibodies binding at the target proteins varied in their applied concentration. The two generated and affinity purified ECL1 and ECL2 had a stock solution of 2 µg/ml. All secondary antibodies that were used for fluorescence stainings also had a stock concentration of 2 mg/ml and were used at 2 µg/ml.

Table 8 summarizes the used primary and secondary antibody combinations, antibody names, clonality, working concentrations of the primary antibodies, order numbers (Order#),
the company they were purchased from, and the fluorescence conjugate for each secondary antibody.

Table 8. Antibodies used in this study

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Associated Secondary Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Clonality</td>
</tr>
<tr>
<td>M-20 Rabbit anti-Fpr3</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>N-20 Rabbit anti-Fpr3</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>ECL1 Rabbit anti-Fpr3</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>ECL2 Mouse anti-Fpr3</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>OMP Rabbit anti-OMP</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Gia Rabbit anti-Gia</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>PDE4A Rabbit anti-PDE4A</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>V2R2 Rabbit anti-V2R2</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>CD45R Rabbit anti-CD45R</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Ly6G Rabbit anti-Ly6G</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Rho Mouse anti-rhodopsin</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>HSV Mouse anti-HSV</td>
<td>Monoclonal</td>
</tr>
</tbody>
</table>

* R. Tirindelli, University of Parma, Parma, Italy; ** Dr. R. Molday, Centre for Macular Research, University of British Columbia, Canada; 1 used for stainings in HEK293T cells; 2 used for stainings on VNO and blood samples; 3 used for colocalization experiments with ECL1; 4 used for colocalization experiments with ECL2

2.6.3.2 Generation of Fpr3 Antibodies

The polyclonal rabbit antibody ECL1 was generated based on the commercially available murine Fpr3 antibody M-20 (see chapter 2.6.3.1). Epitope mapping via peptide-spot assay analysis (see chapter 2.6.3.3) of M-20 revealed two epitopes. For each epitope, a peptide was synthesized and used to immunize a rabbit. Subsequent immunocytochemistry experiments with both rabbit sera on HEK293T cells expressing murine Fpr3 revealed best results for the serum of the rabbit injected with AMKEKWPGWFCLKL. After 12 weeks serum was obtained and antibody was purified by affinity chromatography with the sulfo-linked AMKEKWPGWFCLKL peptide and adjusted to a stock concentration of 2 mg/ml.

The monoclonal mouse antibody ECL2 was developed in cooperation with the Abmart Co. Epitope scoring of the Fpr3 sequence of the mouse was used to determine four peptide sequences (WGNSVEERNTA, LSEDGHSIDTR, HLSSSRLQRALS, and ITTKIHKKAFV) spanning intracellular, extracellular, and C-terminal epitopes on Fpr3. For immunogen production these epitopes were over-expressed in *Escherichia coli* and purified by nickel-affinity chromatography. Each immunogen was then injected into three 8-12 week old female BALB/C mice. For hybridoma cell generation spleen cells from the best
responding mice were fused to SP2/0 myeloma cells and single parent cell colonies were obtained and tested by ELISA for their ability to bind the antigen. The nine most productive and stable clones were injected into the peritoneal cavity of mice. After 10–14 days ascites fluid was obtained and tested. Immunocytochemistry experiments on HEK293T cells expressing murine Fpr3 showed the highest sensitivity for the antibodies produced with the cell line for WGNSVEERLNTA. Antibodies produced by the corresponding hybridoma cell line were used for all ECL2 immunochemistry experiments.

2.6.3.3 Peptide-Spot Array Analysis for Antibody Characterization

For peptide-spot array analysis, 69 peptides covering the whole sequence of murine Fpr3 were synthesized. Each peptide consisted of 15 amino acid residues and overlapped by ten residues with its predecessor. All peptides were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on acid hardened polyvinylidene difluoride (PVDF) cellulose membranes derivatized with a polyethylene glycol spacer. Membranes were equilibrated in 150 mM NaCl, 50 mM Tris/HCl (pH 7.5) for 30 min at RT. Each antibody (M-20, ECL1, and ECL2; see chapter 2.6.3.1) was solved at a concentration of 4 µg/ml in PBS containing 5% (m/v) milk powder. Then they were added to the membrane and incubated overnight at 4°C. After washing with PBS, the membrane was incubated with the corresponding peroxidase-coupled secondary antibody overnight at 4°C. Thereafter, the membrane was washed twice with PBS for 10 min and incubated with enhanced chemiluminescence solution until pronounced signal was visible. Analysis was performed using the Fusion SL (Peqlab) luminescence imaging system. Captured images were used for quantification. Therefore, picture colors were inverted in Photoshop CS5 (Adobe). Each spot was analyzed with a marker circle having a diameter of 3.9 cm (1528 pixels). The average signal value for each spot was then determined with the histogram option.

2.6.3.4 Blocking Peptides

Peptides that were used for antibody generation, AMKEKWPGWFLCKL for ECL1 and WGNSVEERLNTA for ECL2, were also used as blocking peptides. 10 µg/ml of the respective peptide were pre-incubated with antibody for 1 h at RT and then given to the preparation as first antibody application. Thus, the specific binding sites of the antibodies were blocked. Subsequently, all steps of the normal immunostaining protocol were performed (see chapter 2.6.2).
2.7 In Situ Hybridization

2.7.1 Coronal Slices of the Vomeronasal Organ for In Situ Hybridization

Mice were anesthetized with an injection of 2 ml of 6.6% (v/v) Ketamine (Pfizer) and 2.2% (v/v) Rompun® (Bayer) in PBS into the abdominal cavity. They were perfused with ice-cold 4% (m/v) paraformaldehyde (pH 7.4). The VNO was dissected and incubated overnight in 4% (m/v) paraformaldehyde at 4°C. Subsequently, it was incubated in increasing sucrose gradients of 10% (m/v; 2 h), 20% (m/v; 2 h), and 30% (m/v; overnight) at 4°C. Afterwards, the VNO was embedded in Tissue-Tek O.C.T. (Sakura Finetek), frozen in 2-methylbutan that was cooled by liquid nitrogen, and stored at -80°C. For in situ hybridization 12 µm thick slices were cut and collected on microscope slides (Superfrost Plus; Menzel-Gläser).

2.7.2 Design and Generation of RNA Probes

Nucleotide 672 to 1056 from NCBI accession number NM_008042.2 plus 153 bp of the 3’ UTR was used for murine Fpr3 RNA probe design. Additional 153 bp of the 3’ UTR of Fpr3 increased the specificity and minimized overlap with other Fpr genes. Based on these specifications, the DNA template for the Fpr3 probes was amplified with the primers CACTACAAGATTCAAAAAAGCCTTTG and AATATTCTAGGCCCTTTGACTTTTACTTTTTT, and subsequently cloned into a pGEM-T Easy Vector (A1360; Promega) via TA-cloning. Antisense and sense probes were generated by the use of T7 and Sp6 RNA polymerases, respectively. The antisense and sense probes were labeled with Digoxigenin (DIG; Roche) according to the manufacturer’s instructions. Therefore, 1 µg template DNA, 4 µl 5× First Strand Buffer (Invitrogen), 2 µl DTT (0.1 M; Invitrogen), 2 µl 10× DIG-Mix (Roche), 1 µl RiboLock RNase Inhibitor (Thermo Scientific), and 7 µl H2O were mixed and incubated for 90 min at 37°C and taken on ice. Probes were DNase digested by incubation with 2.5 µl 10× DNase buffer (Thermo Scientific) and 1 µl DNase I (50 U/µl; Thermo Scientific) for 15 min at 37°C. Addition of 2.5 µl EDTA (0.2 M; Thermo Scientific) stopped the enzyme reaction. DNase was inactivated by incubation of 10 min at 65°C. Precipitation of the probes was reached by adding 2 µl 4 M LiCl and 60 µl 100% (v/v) ethanol with subsequent overnight incubation at -20°C. Probes were centrifuged at 10,000× g for 20 min at 4°C. Supernatant was discarded and the pellet was washed three times with 100 µl 80% (v/v) ethanol. The RNA pellet was dried and resolved in 25 µl TE buffer (10 mM Tris-Cl pH 7.0, 1 mM EDTA).
**2.7.3 Hybridization**

Coronal VNO slices were thawed and dried for 15 min at RT. They were fixed for 4 min in 4% (m/v) paraformaldehyde and washed twice for 3 min with PBS at RT. Slides were treated with 0.2 M HCl for 10 min at RT and washed twice for 3 min with PBS. Slices were incubated for 10 min with acetylation solution (0.1 M triethanolamine, 1.75 µl/ml acidic acid (99.9% (v/v)), 2.5 µl/ml acetic anhydride) at RT and washed twice for 3 min with PBS at RT. Afterwards, slices were incubated with pre-hybridization solution (50% (v/v) formamide and 600 mM NaCl) for 2 h at 65°C in a sealed chamber filled with formamide. Hybridization solution (50% (v/v) formamide, 10 mM Tris-Cl pH 8.0, 200 µg/ml yeast tRNA, 1× Denhardt’s, 600 mM NaCl, 0.25% (v/v) SDS, 1 mM EDTA pH 8.0) was preheated for 10 min at 85°C. 0.2 ng/µl DIG-labeled probes were added to the preheated hybridization solution and heated for 3 further min. Hybridization solution containing probes was given on the slices and incubated overnight (>12 h) at 65°C in a chamber filled with formamide.

**2.7.4 Washing**

After hybridization, slices were washed once with 5× SSC at 65°C for 30 min, and once with 2× SSC, 0.2 SSC, and 0.1× SSC at RT for 20 min, each. Afterwards, slices were blocked with TN-blocking solution (100 mM Tris pH 7.5, 150 mM NaCl, 2% (v/v) FCS) for 30 min. Slices were incubated with anti-DIG antibody (Roche, 1:5,000) in TN-blocking solution overnight at 4°C. They were washed three times with TN buffer (100 mM Tris pH 7.5, 150 mM NaCl) for 5 min at RT. Slices were equilibrated in AP buffer (100 mM Tris base pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 5 min at RT.

**2.7.5 Detection with Alkaline Phosphatase**

For detection of hybridized RNA, slices were incubated with detection buffer (175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 300 µg/ml nitro blue tetrazolium (NBT), 1 mM levamisole in AP buffer at RT in a chamber filled with TN buffer until the staining was well-developed. The staining reaction was stopped at 4°C and slices were mounted with fluorescence mounting medium (DAKO).


2.8 Software and Web Tools

2.8.1 Software

2.8.1.1 Adobe Photoshop CS5
Adobe Photoshop CS5 (Adobe) was used to adjust brightness and contrast of immuno images. Every pixel in a picture was adjusted in the same way. Images showing staining and respective negative controls were also adjusted in the same way. Pictures were also rotated and cropped in Photoshop.

2.8.1.2 Adobe Illustrator CS6
All figures were created in Adobe Illustrator CS6 (Adobe) to maintain a minimum pixel density of 300 ppi (pixels per inch) that is suitable for high resolution printing.

2.8.1.3 Microsoft Office 2010
This thesis was written and edited in Microsoft Word2010. All quantitative data analyses were performed in Excel2010.

2.8.1.4 VectorNTI Suite 9
VectorNTI Suite 9 (Thermo Scientific) including VectorNTI, ContigExpress, and AlignX was used to design all vector constructs and to analyze chromatographs of sequenced genes. Diverse genealogies for determining sequence relationship between genes were compiled in AlignX.

2.8.1.5 FLIPR system software v2.1.2
Data acquisition and evaluation for FLIPR experiments was performed with FLIPR system software v2.1.2 (Molecular Devices).

2.8.1.6 BD AttoVision™ software v1.6
Data acquisition and evaluation for Bioimager experiments was performed with BD AttoVision™ software v1.6 (BD Biosciences).
**EXPERIMENTAL PROCEDURES**

2.8.1.7 *Graph Pad Prism*

Graph Pad Prism 5.0 (GraphPad Software) was used to calculate concentration-response curves.

2.8.1.8 *Cell^P*

Immuno pictures taken with the Olympus BX61 fluorescence microscope were saved with the Cell^P software.

2.8.2 *Web Tools*

2.8.2.1 *Gene Information Gathering*

The U.S. government-funded national resource for molecular biology information (NCBI, [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)), was used to gather information about genes and their coding regions. Information on the 3’ and 5’ regions was gathered with the Ensembl genome browser ([http://www.ensembl.org/](http://www.ensembl.org/)). Database analyses on which *Fpr3* gene variant is expressed by which mouse strain was performed via consulting the Mouse Genomes Project ([http://www.sanger.ac.uk/science/data/mouse-genomes-project](http://www.sanger.ac.uk/science/data/mouse-genomes-project)).

2.8.2.2 *Prediction of Gene Orthology*

2.8.2.3 Prediction of Transmembrane Domains (TMHMM)

Prediction of the transmembrane domains for murine Fpr3 was performed with the online tool for “Prediction of transmembrane helices in proteins” (TMHMM Server v. 2.0) of the Center for Biological sequence Analysis, Technical University of Denmark (http://www.cbs.dtu.dk/services/TMHMM).

2.8.2.4 Calculation of Primer Melting Temperatures

All melting temperatures of primers were calculated with the Sigma-Genosys DNA Calculator tool (http://www.sigma-genosys.com/calc/DNACalc.asp).

2.8.2.5 Mouse Haplotype Analyses

Haplotype analyses were performed with the Mouse Phylogeny Viewer (http://msub.csbio.unc.edu/) as presented by Yang and colleagues (Yang et al., 2011). According to the Ensembl genome browser, murine Fpr3 is present on chromosome 17 with an exon reaching from nucleotide 17,970,458 to 17,971,677. To include strain specific variations in the gene position, subspecific origins were examined in the nucleotide range from 17,970,000 to 17,972,000.

2.9 Statistics and Mathematics

2.9.1 Average and Standard Deviation

Sample average was calculated with the basic calculation for arithmetic mean for each experiment. Subsequently, the mean values of all associated experiments (e.g. independent transfection or dissociation) were averaged with the basic calculation for the arithmetic mean.

\[
\bar{x} = \frac{1}{n} \sum x_i
\]

Standard deviation (Excel2010; STDEV) was calculated using the formula

\[
\sigma = \sqrt{\frac{1}{n-1} \sum (x - \bar{x})^2}
\]

with \(\bar{x}\) being the sample’s arithmetic mean and \(n\) being the sample size. Resulting error bars show the empirical standard deviation of the sample average. Calculations of sample average and standard deviation were used for quantification of cells in immunocytochemistry- and calcium imaging experiments. Quantification was calculated and evaluated after the same criteria.
2.9.2 Concentration-Response Curves

Curves were calculated with Graph Pad Prism 5.0 (GraphPad Software) using the equation

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{\left(\frac{\text{LogEC50} - X}{\text{HillSlope}}\right)}},
\]

for sigmoidal concentration-response with variable slope, with “Bottom” being the Y value at the bottom plateau, “Top” being the Y value at the top plateau, LogEC50 being the X value when the response is halfway between Bottom and Top, and the Hill-Slope describing the steepness of the curve. For statistical analysis, only curves with calculated R^2-values > 0.95 were used. The absolute sum of squares for normalized curves was < 900. The highest ligand concentration used to create concentration-response curves was 30 µM. Empirical average and standard deviation were calculated from independent measurements.
3 RESULTS

3.1 Generation and Characterization of Two Novel Fpr3 Antibodies

An immunofluorescence analysis with different antibodies was performed to gain new insight into the murine Fpr3 expression pattern. The goal was to study Fpr3 expression in different mouse tissues via immunohisto- and cytochemistry. First, two commercially available polyclonal antibodies specifically designed against Fpr3 were tested. It was important that they specifically detected Fpr3 and did not cross-react with other proteins, particularly with close members of the murine Fpr family. Therefore, I first characterized the two antibodies in a heterologous expression system using HEK cells transiently transfected with murine Fpr3 as an established standard laboratory procedure (Bufe et al., 2012).

3.1.1 Characterization of Commercially Available Fpr3 Antibodies

To test if both commercially available Fpr3 antibodies, M-20 (sc-18195; Santa Cruz Biotechnology, Inc.; see chapter 2.6.3.1) and N-20 (orb100776; Biorbyt; see chapter 2.6.3.1), specifically bind Fpr3, they were applied to HEK cells transiently transfected with the receptor at dilutions of 1:50–1:500.

First, I tested the N-20 antibody for its specific detection of murine Fpr3 (Figure 9). Cells were transfected with Fpr3 attached to an N-terminal rhodopsin-epitope (Rho-tag) (Figure 9A). The Rho-tag was detected with an antibody directed against this epitope, which controlled for expression of the receptor. Representative images of N-20 showed no staining at any of the tested dilutions (Figure 9B). Even the highest concentration 10 µg/ml (1:50) showed no specific signal. Cells were transfected with the empty vector (mock) as a negative control. The positive Rho-tag stained control showed pronounced Fpr3 expression, despite no staining with the N-20 antibody.

Figure 9. Test of the commercially available Fpr3 antibody N-20. A. model of the murine G protein coupled receptor Fpr3 with its seven transmembrane domains, three external and three internal loops, and N- and C-terminus. The exact epitope for N-20 on Fpr3 is proprietary to Biorbyt. It is located within the range of the residues 61 and 160 and is marked with an orange line. The N-terminally attached Rho-tag is indicated in purple. B, immunocytochemistry on HEK293T cells transiently expressing either Fpr3 or an N-terminal Rho-tagged
version of the receptor performed with the polyclonal rabbit antibody N-20 and a Rho-tag antibody. Shown are representative images of N-20 at 10 µg/ml (1:50) and of the Rho-tag antibody used 1:500. mFpr = murine Fpr. Scale bar, 20 µm.

This result demonstrates a lack of proper detection of Fpr3 using N-20, although the antibody was originally designed to detect this receptor. Therefore, the N-20 antibody was not suitable for investigating Fpr3 expression.

Representative immunostainings using the M-20 antibody showed specific Fpr3 staining for M-20 up to 0.4 µg/ml (1:500) (Figure 10). The signal was only visible at high antibody concentrations but suffered from a high signal to noise ratio. Staining intensity decreased rapidly at lower antibody concentrations (Figure 10A). Quantification revealed a drastic drop from 9.3% ± 1.4% to 2.9% ± 1.7% stained cells from the 4 µg/ml (1:50) to 2 µg/ml (1:100) dilutions, and almost no specific signal was detected at 0.4 µg/ml (1:500). Moreover, the M-20 antibody showed high non-specific staining that increased at higher antibody concentrations, and the specific signal was only two-fold higher than that of the non-specific background at 4 µg/ml (1:50) (Figure 10B), which would have been the best concentration for immunohistochemistry. This was not sensitive enough for use on native cells.

These results show that M-20 was limited to an analysis of murine Fpr3 in a heterologous overexpression system and demonstrate that neither of the commercially available Fpr3 antibodies was suitable for examining the Fpr3 protein in native cells.

![Image](image_url)

Figure 10. Characterization of the commercially available Fpr3 antibody M-20. Immunocytochemistry on murine Fpr3 and mock (empty vector) transfected HEK293T cells with the polyclonal rabbit antibody M-20. A, representative images of the dilutions 1:50 and 1:200. Scale bar, 20 µm. B, quantification of the average background intensity in relative fluorescence units (RFU) for all used concentrations of M-20. mFpr = murine Fpr.

### 3.1.2 Generation of Fpr3 Antibodies

Because neither commercially available Fpr3 antibody produced satisfying results, I decided to generate own antibodies for the detection of murine Fpr3. I planned the production of two antibodies to increase the chances of successfully establishing one or more reliable
Fpr3 antibodies. The first antibody was a polyclonal Fpr3 antibody made in rabbits based on the commercial M-20 antibody. However, it should have improved sensitivity and should produce less background staining. Because an efficient Fpr3 antibody was very important for this work, I generated a monoclonal mouse antibody directed against Fpr3, in parallel. This should maximize the chances of producing a functioning antibody that can be used for stainings in native tissue. Different species of origin should allow greater versatility in the design of later experiments and to bypass species-related background issues. The monoclonal antibodies were prepared in cooperation with the Abmart Co. in a mouse hybridoma cell line.

3.1.2.1 Generation of Polyclonal Rabbit Fpr3 Antibodies

An epitope map of the M-20 antibody was prepared by peptide-spot array analysis (see chapter 2.6.3.3) in cooperation with Dr. Martin Jung to identify suitable epitopes on murine Fpr3 to produce the immunization peptide. A set of 69 peptides, including all 351 amino acid residues of murine Fpr3, was prepared and spotted on a membrane. Each peptide was 15 amino acids in length and overlapped 10 residues with the preceding peptide. The antibody was applied to the synthesized peptides at a 1:50 dilution (Figure 11).

The peptide-spot assay revealed several epitopes on the N-terminus, C-terminus, transmembrane domains, and extracellular loops of murine Fpr3 (Figure 11A). Only the epitopes on the extracellular loops were of interest because the Fpr family has the strongest sequence diversity in these areas. The main peptide fragments AMKEKWPFGWFLCKL and MQFSGSYKIIIGRLVN in the Fpr3 extracellular loops were detected by the M-20 antibody (Figure 11B, C). Two rabbits were immunized with each peptide, and the sera were applied to HEK cells transfected with Fpr3 over 16 weeks. The antibody sera obtained from the animal immunized with the AMKEKWPFGWFLCKL peptide showed specific staining that increased weekly, whereas the sera obtained with MQFSGSYKIIGRLVN did not produce any staining. Thus, the AMKEKWPFGWFLCKL serum was collected. The antibody was purified by affinity chromatography (see chapter 2.6.3.2) to increase sensitivity and decrease non-specific staining. The resulting polyclonal rabbit antibody directed against murine Fpr3 was called ECL1 because its peptide for immunization, AMKEKWPFGWFLCKL, is present on the first extracellular loop of the receptor (in short ECL1). A subsequent immunocytochemical analysis confirmed that ECL1 was more sensitive than M-20.
RESULTS

Figure 11. **Epitopes of the commercially available Fpr3 antibody M-20.** Peptide spot array analyses of M-20. 
A, original array visualized by enhanced chemiluminescence. 
B, the commercially available Fpr3 antibody M-20 was tested against peptides comprising the complete sequence of murine Fpr3. Each spot consists of a 15 amino acid peptide overlapping by ten residues with its predecessor. Therefore, parts of every 15 amino acid motif are present in five spot-peptides. Shown are the peptide sequences that comprise both main epitopes on Fpr3. 
C, model of murine Fpr3 with its seven transmembrane domains, three external and three internal loops, and N- and C-terminus. The two epitopes identified for M-20 on the first and third extracellular loop are marked with green (AMKEKWPGFWGFLCKLC) and blue (MQFSGSYKIGRLVN) lines. Peptide spot arrays were performed by Dr. Martin Jung, Department of Medical Biochemistry and Molecular Biology, Saarland University. mFpr = murine Fpr.

### 3.1.2.2 Generation of Monoclonal Mouse Fpr3 Antibodies

Nine mouse antibodies were generated in hybridoma cell lines from BALB/c mice based on four peptide fragments from murine Fpr3 whose sequences had low similarities with the sequences of the other six members of the murine Fpr family. Epitopes were chosen based on peptides with a possible low consensus of Fpr3 with the other six members of the murine Fpr family. The four selected Fpr3 epitopes were WGNSVEERLNTA from the second extracellular loop, ITTKIHKKAFCV from the third intracellular loop, and HSLSSRLQRALS and LSEDSGHSIDTR from the C-terminus. Three antibodies were generated with the first peptide (ECL2-1, ECL2-2, and ECL2-3), one with the second (ICL3), three with the third (CT1-1, CT1-2, and CT1-3), and two with the fourth (CT2-1 and CT2-2) peptide (Table 9).

To figure out which antibody was best suited to examine murine Fpr3 expression, I performed immunocytochemistry experiments with all nine antibodies at 1 µg/ml on Fpr3 and mock transfected HEK cells (Figure 12).

ICL3, CT2-1, and CT2-2 showed no staining for Fpr3, whereas ECL2-1, ECL2-2, ECL2-3, CT1-1, CT1-2, and CT1-3 clearly detected the receptor (Figure 12A). Quantification revealed that ECL2-1 and ECL2-2 were the most sensitive of the nine antibodies (Figure 12B). They detected 11.7% ± 0.6% and 11.6% ± 2.1% of the cells, respectively. ECL2-3, CT1-1, CT1-2, and CT1-3 only detected 5.1% ± 0.6% to 7.1% ± 2.3% of the Fpr3-expressing cells. Because
ECL2-1 combined the best staining intensity with the lowest background in the mock, it was used for further experiments and called ECL2.

Figure 12. **Characterization of monoclonal mouse Fpr3 antibodies.** Immunocytochemistry on HEK293T cells transfected with murine Fpr3 with the nine monoclonal mouse antibodies (1:2,000). **A**, representative images of all nine monoclonal mouse antibodies. Scale bar, 20 µm. **B**, Quantification of the average stained cells for each antibody. Numbers in parentheses denote positive versus total cells. Error bars, S.D.

In summary, these data demonstrate that the ECL1 polyclonal rabbit and ECL2 monoclonal mouse antibodies stained in a heterologous overexpressing system. However, it was unclear whether these antibodies would work in complex immunohistochemistry reactions in the VNO or on immune cells, as many other receptors and possible targets for non-specific antibody binding are present in native tissues. To further consolidate the functionality and specificity of the Fpr3 antibodies, they were characterized in HEK cells on Fpr3 and all other members of the murine Fpr family.
Table 9. Peptide fragments for the generation of monoclonal Fpr3 antibodies

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Position</th>
<th>Amino Acids</th>
<th>Location</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>WGNSVEERLNTA</td>
<td>183-194</td>
<td>183-194</td>
<td>2nd extracellular loop</td>
<td>ECL2-1, ECL2-2, ECL2-3</td>
</tr>
<tr>
<td>ITTKIHKKAIV</td>
<td>224-235</td>
<td>224-235</td>
<td>3rd intracellular loop</td>
<td>ICL3</td>
</tr>
<tr>
<td>HSLSSRLQRALS</td>
<td>315-326</td>
<td>315-326</td>
<td>C-terminal</td>
<td>CT1-1, CT1-2, CT1-3</td>
</tr>
<tr>
<td>LSEDSGHISDTR</td>
<td>325-336</td>
<td>325-336</td>
<td>C-terminal</td>
<td>CT2-1, CT2-2</td>
</tr>
</tbody>
</table>

3.1.3 Characterization of Two Novel Fpr3 Antibodies

ECL1 and ECL2 were generated with peptides representing non-overlapping sites of murine Fpr3. Their epitopes were on two different extracellular loops of the receptor, which possess the highest sequence divergence among the members of the murine Fpr family. Antibodies can occasionally recognize more than one domain within a protein. Multiple recognition sites and unfavorable epitopes can increase the chance for non-specific binding or cross-reactivity. The binding sites on murine Fpr3 were characterized to ensure that ECL1 and ECL2 only recognized the epitopes used for their generation (Figures 13, 14).

First, a peptide-spot array analysis was performed to precisely determine the recognition sites of both antibodies (Figure 13). ECL1 and ECL2 were tested at 4 µg/ml (1:500) on 69 peptides covering the entire murine Fpr3 sequence spotted on a membrane, as described previously (see chapter 3.1.2).

Both antibodies showed strong immunoreactivity to the sequences used for their generation (AMKEKWPFGWFLCKL and WGNSVEERLNTA) and only weakly interacted with other receptor domains. The secondary antibody controls did not react with any of the domains. Parts of the peptide sequences were present in five of the 69 spotted peptides due to a five amino acid shift from the peptide to its predecessor (Figure 13). However, the antibodies did not react with all five peptides. Each antibody bound strongly to only one spot. Surprisingly, the particular spot did not comprise the full-length peptide used for
imunization. Instead, only a small portion of the respective original peptide was bound by the antibodies and led to strong staining in the peptide-spot assay. The peptide-spot containing the AMKEK C-terminal showed the highest staining intensity for ECL1, whereas the peptide containing the LNTA N-terminal showed the strongest reaction for ECL2. A comparison between signal intensities and the peptide sequences revealed that the key ECL1 and ECL2 binding residues were AMKEK and LNTA, respectively (Figure 13A, B). Surprisingly, both motifs were also contained in neighboring peptide spots to which the antibodies showed no strong immunoreactivity, indicating recognition of only a very specific conformation of the respective peptide epitopes for both antibodies. A database analysis of approximately 20,000 mouse genes (Church et al., 2009) revealed the presence of the AMKEK and LNTA motifs in only 0.08% (17) and 0.6% (119) of all mouse genes, respectively (Tables 11 and 12; Appendix). Thus, both motifs are reasonably specific for murine Fpr3. Moreover, they were absent in most Fprs of the mouse, except Fpr2, which has 88.5% sequence identity with Fpr3.

Figure 13. Epitopes of the two novel specific Fpr3 antibodies ECL1 and ECL2. Peptide spot array analyses of the polyclonal rabbit antibody ECL1 (A, green) and the monoclonal mouse antibody ECL2 (B, red). Models indicate the positions and sequences of the immunization peptides used for antibody generation. Both antibodies were tested against peptides comprising the complete sequence of murine Fpr3. Each spot consists of a 15 amino acid peptide overlapping by ten residues with its predecessor. Insets show original arrays visualized by enhanced chemiluminescence. The charts show the quantification of staining intensities; bars containing the AMKEK or LNTA motif are colored. Numbers denote the peptide positions in the receptor protein; the part of the immunization peptide sequences that are recognized by the antibodies are colored.

The antibodies were applied to HEK cells transiently transfected with either Fpr3 or another member of the murine Fpr family to test whether the antibodies cross-reacted with Fpr2 or any other murine Fpr (Figure 14).

Both antibodies recognized Fpr3-expressing cells in a nearly identical manner and stained exclusively for this receptor. No cross-reactivity to any other member of the murine Fpr family, such as Fpr1, Fpr2, Fpr-rs3, Fpr-rs4, Fpr-rs6, or Fpr-rs7, was detected. Moreover,
RESULTS

No non-specific staining was observed on any of the HEK cells transfected with these receptors. Quantification revealed a nearly identical percentage of cells stained with ECL1 and ECL2 (Figure 14B). ECL1 stained 11.6% ± 2.7% and ECL2 stained 11.2% ± 1.7% of the Fpr3 transfected cells. Although all receptors were well-expressed in HEK cells, I did not observe any specific reactivity to Fpr2 or any other murine Fpr.

![Figure 14. ECL1 and ECL2 detect Fpr3 and do not recognize other murine Fpr family members. A, immunostaining of HEK293T cells to test the cross-reactivity of ECL1 (upper panel) and ECL2 (lower panel) for the indicated receptors. Insets show stainings with an anti-rhodopsin-epitope (Rho) as a positive control for expression of the given receptors. Scale bars, 20 µm. B, averaged percentage of stained cells from three independent experiments. Numbers in parentheses denote positive versus total cells. mFpr = murine Fpr. Error bars, S.D.](image)

Both newly generated antibodies were specific for murine Fpr3. Next, I was interested in determining whether one antibody was more sensitive than the other. Therefore, the sensitivities of both antibodies were tested by serial dilutions from 20 µg/ml (1:100) to 0.002 µg/ml (1:1,000,000) on HEK cells expressing Fpr3 (Figure 15).

Reactivity of the polyclonal ECL1 was optimal up to 2 µg/ml (1:1,000), but decreased with further dilution (Figure 15A). ECL1 stained 61% and 44% of the cells at 0.67 µg/ml (1:3,000) and 0.2 µg/ml (1:10,000), respectively. Further dilution showed very little to no staining. The antibody had no reactivity over background at ≤0.02 µg/ml (1:100,000). The monoclonal ECL2 antibody was even more sensitive and showed full activity up to 0.2 µg/ml (1:10,000) (Figure 15B). Further dilution revealed little staining. ECL2 had reactivity of 63% at 0.067 µg/ml (1:30,000), which decreased to background at ≤0.02 µg/ml (1:100,000).
Taken together, these results demonstrate the specificity and sensitivity of the newly developed Fpr3 antibodies, indicating their suitability for immunohisto- and cytochemical experiments with native tissues.

![Figure 15. ECL1 and ECL2 show high sensitivity for Fpr3. Antibody dilution experiment examining the sensitivity of ECL1 (A) and ECL2 (B) in HEK293T cells tested in decreasing concentrations from 1:100 to 1:1,000,000. Signals were normalized to 1:100 dilution. Immunoreactivity was compared to mock transfected cells (gray). mFpr = murine Fpr.](image)

3.2 Fpr3 Expression in the Murine Vomeronasal and Immune Systems

The expression pattern of murine Fpr3 throughout the body is only partially understood. Expression of this receptor is best understood in the vomeronasal system, whereas in other tissues Fpr3 expression is currently subject of controversy (see chapter 1.3.3). Two independent studies (Liberles et al., 2009, Rivière et al., 2009) provided clear evidence for Fpr3 expression in a small subpopulation (< 1%) of vomeronasal sensory neurons by quantitative RT-PCR and in situ hybridization. However, direct detection of Fpr3 protein is still missing. With the new Fpr3 antibodies ECL1 and ECL2, I planned to examine Fpr3 expression in the VNO and immune system of the mouse.

3.2.1 Fpr3 Protein is Expressed in Vomeronasal Sensory Neurons and in Immune Cells

For the detection of murine Fpr3 protein in native cells I initially wanted to focus on a well-studied tissue. I decided that the VNO should be excellently suited to first examine Fpr3 expression with the novel antibodies based on the consistent reports on Fpr3 expression and the challenging amount of target cells. To be absolutely sure about the presence of Fpr3 in the VNO, I used in situ hybridization and RT-PCR to independently prove expression of Fpr3 mRNA (Figure 16).

With RT-PCR experiments a band of the correct size for murine Fpr3 from VNO cDNA was reproducibly \((n = 5)\) detected (Figure 16A). The control reaction lacking reverse
RESULTS

transcriptase showed no band at all. Sequencing indeed revealed *Fpr3* as the amplification product. Next, *in situ* hybridization was performed to achieve cellular resolution (Figure 16B). In line with previous reports (Liberles et al., 2009, Rivière et al., 2009) *Fpr3* mRNA in a small subset of VSNs from C57Bl/6NCrl mice was observed in the antisense reaction. The sense reaction showed no specific signal.

![Figure 16. Fpr3 mRNA is expressed in the vomeronasal organ. A, RT-PCR analysis of murine Fpr3 expression in the vomeronasal organ (VNO). A band of the correct size and sequence was observed in VNO cDNA (+RT) but not in the negative control lacking reverse transcriptase (-RT). Similar results were obtained in five independent experiments. (L) FastRuler Middle Range DNA Ladder. B, in situ hybridization with sense and antisense probes for Fpr3 on coronal slices of the vomeronasal organ. Black triangles mark Fpr3-positive cells. mFpr = murine Fpr. Scale bar, 100 µm.](image)

After having confirmed the presence of *Fpr3* mRNA in the VNO, expression of Fpr3 protein in dissociated VNO cells was examined (Figure 17). For these experiments, the monoclonal mouse antibody ECL2 was used because of its superior sensitivity over the polyclonal ECL1 antibody (Figure 15).

![Figure 17. Fpr3 protein is expressed by a small subset of vomeronasal cells. A, representative immunostaining with ECL2 on dissociated vomeronasal cells of C57Bl/6NCrl mice. The white triangle marks an Fpr3-positive cell (red). B, quantification of murine Fpr3 immunoreactivity. Average frequency of Fpr3 expression was analyzed in a total of 60,426 cells from six independent experiments. Antibody specificity was demonstrated by blocking the specific binding site through preincubation with 10 µg/ml of the peptide used for antibody generation. Error bars, S.D.](image)
RESULTS

Staining reactions with ECL2 produced a clear and convincing signal in a subset of VNO cells (Figure 17A). Only very little non-specific staining was visible. Quantitative analysis of 60,426 cells from seven independent experiments revealed Fpr3 expression in 188 cells, which corresponds to an average expression rate of 0.3% (Figure 17B). Specificity of the staining was controlled by the blocking peptide.

mRNA of murine Fpr3 has been found in sensory neurons of the basal VNO layer that also express GaO (Liberles et al., 2009) but are negative for the basal Vmn2rs and the apical marker Ga2 (Liberles et al., 2009). With regard to these findings, I performed colocalization immunocytochemistry experiments with the ECL2 antibody and antibodies for the cellular marker GaO, Vmn2r1 (V2R2), a marker for Vmn2r-positive VSNs (Martini et al., 2001), and phosphodiesterase 4A (PDE4A), a molecular marker of apical VSNs (Lau and Cherry, 2000) (Figure 18).

According to the previous report (Liberles et al., 2009), I expected coexpression with GaO and none with PDE4A or V2R2. First, colocalization of Fpr3 with PDE4A and Vmn2rs was examined. The expression pattern for Fpr3 protein in my experiments was consistent with my expectation as there was virtually no colocalization between Fpr3 and PDE4A or V2R2. As expected, a pronounced overlap for Fpr3 and GaO protein was detected. Quantification revealed colabeling in 56% (61/108) of the cells. Because of the assumption that the GaO-positive cells were mature VSNs that also expressed olfactory marker protein (OMP), which is present in all mature VSNs (Margolis, 1982), the experiment was repeated with this particular protein. Staining for OMP confirmed the result obtained for GaO, as pronounced coexpression for Fpr3 and OMP was visible. 54% (93/171) of the Fpr3-positive cells coexpressed with the marker for mature VSNs. Interestingly, only about half of the Fpr3-positive cells coexpressed with the markers that labeled them as VSNs. Hence, nearly half of the cells positive for Fpr3 in this preparation did not express specific markers of mature VSNs (GaO and OMP), indicating that they could comprise non-olfactory cell types.
RESULTS

Figure 18. Characterization of Fpr3-positive vomeronasal cells. Colocalization of murine Fpr3 (red) with different cellular markers (green) on dissociated vomeronasal cells from C57BL/6NCrl mice. Fpr3 staining colocalized with phosphodiesterase 4A (PDE4A), as a marker for the apical zone of the VNO, vomeronasal type 2 receptor 1 (V2R2), as a marker for V2R2 expressing cells, the G protein alpha o subunit (Gαo), as a marker for the basal zone, and the olfactory marker protein (OMP), as a marker for mature vomeronasal sensory neurons. The colocalizing cells are marked with a white triangle, non-colocalizing with a white arrow. All cells were counterstained with the nuclear dye Hoechst33342 (blue). Insets show Fpr3-positive cells in a 3× magnification. Scale bars, 20 µm. The bar chart below each picture denotes the quantification from four to five experiments, analyzing the colocalizations in a total of 25,000 to 57,000 cells. Precise numbers are given in the graphs. Fpr3-positive cells that coexpress the marker are labeled by (+), while cells not coexpressing the marker are labeled with (-). Values in parentheses denote positive versus total cells. Error bars, S.D.

Consistent with these findings, there are hints in the literature for murine Fpr3 expression in immune cells. Thus, I assumed that the OMP- and Gαo-negative cells may have been leukocytes. As my cell preparation was likely to contain trace contaminations from white blood cells, I hypothesized that the Fpr3 antibody detected some leukocytes in addition to VSNs. To evaluate this, coexpression of two immune cell markers with Fpr3 was tested (Figure 19). An antibody directed against the lineage-specific R-isoform of the cluster of differentiation molecule 45 (CD45R), a plasma membrane phosphatase and one of the most commonly used pan-B cell markers, which is expressed in most leukocytes, was used. However, this molecule is not expressed by neutrophil granulocytes. Thus, for detection of this cell type, an antibody detecting the lymphocyte antigen 6G (Ly6G), a marker exclusively expressed by neutrophil granulocytes, was used (Ballas and Rasmussen, 1993; Rolink et al., 1996; Lai et al., 1998).

Both immune cell marker antibodies produced clear and robust stainings. Expression of Ly6G was examined first because it is only expressed by one cell type and would therefore deliver more specific results. For this molecule, marked overlap of Fpr3 and Ly6G protein was observed in 28 of 64 (44%) of the analyzed cells. Subsequently, CD45R expression was
examined to evaluate if any other immune cell type also expressed murine Fpr3. Virtually no coexpression with Fpr3 was detected for CD45R. These results provide first evidence for a dual expression of Fpr3 protein in specific subsets of neurons in the VNO and in immune cells and prove expression of Fpr3 mRNA and protein in murine vomeronasal sensory neurons that also express Goαo and OMP.

Figure 19. Molecular characteristics of the non-olfactory Fpr3-positive cells. Colocalization of murine Fpr3 (red) with immune cell markers (green) on dissociated VNO cells. Fpr3 staining colocalized with Ly6G (lymphocyte antigen 6G), a neutrophil granulocyte marker. No colocalization was seen for CD45R (cluster of differentiation molecule 45R), that is expressed by most immune cells but absent in neutrophil granulocytes. The colocalizing cells are marked with a white triangle, non-colocalizing with a white arrow. All cells were counterstained with the nuclear dye Hoechst33342 (blue). Insets show Fpr3-positive cells in a 3× magnification. Scale bars, 20 µm. The bar chart below each picture denotes the quantification from two to five experiments, analyzing the colocalizations in a total of 7,000 to 17,000 cells. Precise numbers are given in the graphs. Values in parentheses denote positive versus total cells. Error bars, S.D.

3.2.2 Fpr3 Expression in Neutrophil Granulocytes is Enhanced by LPS Stimulation

Mouse blood cells were analyzed by immunocytochemical and RT-PCR to characterize Fpr3-positive immune cells in greater detail. First, the newly generated antibodies were used to directly test leukocytes from murine blood smears for Fpr3 expression (Figure 20).

ECL2 and ECL1 produced clear stainings on nucleated blood cells with little to no non-specific staining (Figure 20A, B; left panel). Staining was observed in 182/1377 leukocytes for ECL2 and in 907/7844 leukocytes for ECL1, resulting in mean percentages of 13.4% ± 0.6% and 13.2% ± 2.6%, respectively (Figure 20C). Furthermore, the signals of both antibodies colocalized to 89.0% ± 4.8% (Figure 21C). The stainings of both antibodies could be abolished by blocking of the epitope specific antibody binding sites with the peptides that were used for their generation. The number of Fpr3-expressing leukocytes corresponded well to the typically range of neutrophil granulocytes in mouse blood, which is between 9% to 18% (Gowen and Calhoun, 1943). Moreover, Fpr3-positive leukocytes showed a multi-lobed nucleus that is typical for mature neutrophil granulocytes (Figure 20A, B; right panel). This result suggests that the Fpr3-expressing leukocytes are neutrophil granulocytes.
RESULTS

Figure 20. **Fpr3 is expressed in mouse leukocytes.** Representative immunostainings of blood cells from C57Bl/6NCrl mice with the monoclonal mouse antibody ECL2 (**A**) or the polyclonal rabbit antibody ECL1 (**B**). For nuclear staining Hoechst33342 was used (right). Scale bars, 10 µm. **C**, quantification and specificity of ECL2 and ECL1 immunoreactivity in leukocytes. Antibody specificity was demonstrated by blocking the specific binding site through preincubation with 10 µg/ml of the peptide used for antibody generation.

To prove this, I performed colocalization experiments between murine Fpr3 and the neutrophil granulocyte marker Ly6G (Figure 21). They should have given definite information about whether these Fpr3-expressing cells are neutrophil granulocytes.

Staining with the Ly6G antibody colocalized with Fpr3 protein expression determined with ECL2 and ECL1 (Figure 21A, B). Quantification revealed colabeling between Ly6G and ECL1 or ECL2 of 83.1% ± 9.3% and 86.7% ± 3.1%, respectively (Figure 21C). Hence, these results unambiguously demonstrate the presence of Fpr3 protein in mouse neutrophil granulocytes.

Figure 21. **Fpr3 protein is expressed in mouse neutrophil granulocytes.** Colocalization between the monoclonal mouse antibody ECL2 (**A**) and the polyclonal rabbit antibody ECL1 (**B**) with the neutrophil granulocyte marker Ly6G. Cell nuclei are shown in blue. Scale bar, 5 µm. **C**, colocalization between Fpr3 and Ly6G immunoreactivity in leukocytes. Bars show average percentage of colocalizing cells from at least three independent experiments. Numbers in parentheses denote positive versus total cells. Error bars, S.D.
RESULTS

In line with the presence of Fpr3 protein in mouse blood cells, Fpr3 mRNA should have also been detectable in those cells. First, PCR experiments were performed to investigate Fpr3 mRNA expression in blood cells (Figure 22).

Surprisingly, no band proving Fpr3 mRNA in mouse blood was visible. To exclude technical problems, a number of positive controls were performed. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), an essential enzyme in glycolysis that is present in all cells and control for cDNA quality, was readily amplified from blood and vomeronasal cDNA. Furthermore, a band for Fpr3 from vomeronasal cDNA was easily detected. In general, mRNA from blood cells was detectable without a problem and therefore I could largely exclude any technical problems as the reason for this unexpected result. A possible explanation for this rather surprising result was the relatively low RNA amount in mature neutrophil granulocytes circulating in the blood. Indeed, these cells possess 10-fold to 20-fold less RNA per cell than monocytes or lymphocytes (Cassatella, 1999). Thus, I concluded that the detection limit of the RT-PCR was too low to amplify Fpr3 from neutrophil granulocyte RNA.

In blood only mature neutrophil granulocytes are present. Their maturation and proliferation takes place in the bone marrow. Hence, it was possible that the mRNA levels for Fpr3 were much higher in maturing neutrophil granulocytes. Moreover, it is well-known that mRNA levels of specific genes in leukocytes, such as Fpr1 (Mandal et al., 2005) and Fpr2 (Cui et al., 2002; Iribarren et al., 2003), increase after stimulation with bacterial stimuli. Thus, I assumed that this could also be true for Fpr3. To test this, bone marrow cells were
RESULTS

stimulated with lipopolysaccharide (LPS) from *Salmonella enteriditis* prior to mRNA isolation and both preparations were examined for Fpr3 expression (Figure 23).

LPS stimulation of bone marrow cells in RT-PCR experiments fulfilled my assumptions. When stimulating cells with 150 µg/ml LPS from *Salmonella enteriditis*, Fpr3 mRNA expression was detected in all LPS-stimulated samples (Figure 23; *left panel*). A strong band for murine *Fpr3* was amplified. Controls with β-actin, a cytoskeletal protein present in each cell, and without reverse transcriptase showed the expected results. In line with my hypothesis, a band for *Fpr3* from mRNA of unstimulated bone marrow cells was not observed (Figure 23; *right panel*), although the controls showed the desired results. Hence, Fpr3 mRNA levels in unstimulated cells were relatively low and LPS stimulation induced an increase in *Fpr3* expression, which explained why most previous RT-PCR studies failed to detect it. These results show that murine Fpr3 is upregulated upon LPS stimulation.

![Figure 23](image1.png)

**Figure 23.** *Fpr3* mRNA expression is induced by LPS stimulation. RT-PCR analysis of murine Fpr3 expression in bone marrow. Receptor expression was observed upon stimulation with 150 µg/ml lipopolysaccharide (LPS) from *S. enteriditis* (left). Fpr3 was not detected from unstimulated mouse bone marrow cDNA (right). β-actin was amplified from all cDNAs. Similar results were obtained in two independent experiments. Size marker (L) FastRuler Middle Range DNA Ladder. mFpr = murine Fpr.

Assuming a similar biological role for murine and human FPR3, I was interested in determining whether both receptors are present in the same cell type. Because of the successful detection of murine Fpr3 in neutrophil granulocytes, I next investigated human FPR3 expression in this cell type (Figure 24). RT-PCR experiments with human neutrophil granulocytes that were stimulated with 150 µg/ml LPS from *Salmonella enteriditis* showed human *FPR3* mRNA expression. A marked band for human *FPR3* was amplified. Positive controls with Gapdh, human *FPR1*, and human *FPR2* that have been reported to be
expressed in neutrophil granulocytes (see chapter 1.3.3) all showed distinct bands. Negative controls without reverse transcriptase showed no bands. These results demonstrate human FPR3 expression in neutrophil granulocytes.

**Figure 24. Human neutrophil granulocytes express three Fprs.** RT-PCR analysis of FPR1, FPR2, and FPR3 expression in human neutrophil granulocytes. Bands of the correct sizes and sequences for all three receptors were observed in human neutrophil granulocyte cDNA (+RT) but not in the negative control without reverse transcriptase (-RT). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as reverse transcription control. Similar results were obtained in three independent experiments. Size marker (L) FastRuler Middle Range DNA Ladder. hFPR = human Fpr.

I reasoned that the increase in RNA through LPS stimulation indicates increased levels of receptor protein, which should have been detectable in corresponding immunocytochemistry experiments. Thus, unstimulated and LPS-stimulation mouse bone marrow cells were examined using ECL2 to detect murine Fpr3 protein (Figure 25).

ECL2 produced reliable stainings on bone marrow cells, as before on blood cells (Figure 25A). Staining was visible in unstimulated and LPS-stimulated cells. Representative images depicted increased cell amounts positive for Fpr3 after LPS stimulation. Quantification revealed that the number of Fpr3-positive cells indeed almost doubled from 5.1% ± 0.7% to 9.5% ± 0.3% (Figure 25B). Thus, expression of Fpr3 protein in mouse neutrophil granulocytes can be induced by LPS.

**Figure 25. Fpr3 protein levels rise with increasing mRNA levels upon LPS stimulation.** A, representative immunostainings for Fpr3 in unstimulated (left) and LPS stimulated (right) bone marrow cells from C57Bl/6NcrI mice. B, quantification for stainings on both conditions. Scale bar, 20 µm. Bar chart shows mean increase of Fpr3-expressing cells upon LPS stimulation from two independent experiments carried out as triplicates. Numbers in parentheses denote positive versus total cells. Error bars, S.D.
Taken together, these results prove mRNA expression for murine and human Fpr3 in neutrophil granulocytes and the presence of murine Fpr3 protein in this cell type. They also demonstrate upregulation of murine Fpr3 mRNA expression upon LPS-stimulation.

3.3 Strain-Specific Variants of Murine Fpr3

Two previous studies (Gao et al., 1998; Wang and Ye, 2002) reported divergent sequences for murine Fpr3 between BALB/c and 129/S6 mice, with a main difference of four amino acids missing in the fourth transmembrane region. Data from these reports suggest strain-specific variants of the receptor that could result in altering expression patterns. The experiments described thus far were performed in C57Bl/6NCrl mice. To investigate potential altering expression patterns of Fpr3, some of the previously described experiments were also performed in other mouse strains. In line with published data, I received varying staining patterns for Fpr3 in different mouse strains, indicating diverging expression patterns for the receptor in different mouse strains. However, initial examinations on this issue lacked a systematic approach.

3.3.1 Fpr3 Protein Expression Occurs in a Strain-Specific Manner

To test the hypothesis of murine Fpr3 variations present in different mouse strains which possibly lead to strain-specific variations in the Fpr3 expression pattern, I performed a systematic expression and genotype analysis of Fpr3 in five mouse strains and combined these results with sequence data from 32 laboratory and nine wild-derived strains. First, 129X1/Sv mice were analyzed via immunohistochemistry using ECL2 and ECL1 (Figure 26).

Remarkably, 129X1/Sv mice showed no Fpr3 expression in leukocytes, whereas C57/Bl6NCrl mice showed Fpr3 expression as before (Figure 26A). Fpr3 expression was also examined in vomeronasal cells of the two mouse strains to prove the result obtained in leukocytes. In vomeronasal cells from 129X1/Sv mice, Fpr3 was not detected, whereas examinations in C57/Bl6NCrl mice resembled the already shown results. In these experiments, ECL2 and ECL1 were used (Figure 26B), and the obtained result was highly reproducible in multiple animals (n = 9).
Figure 26. **Strain-specific loss of Fpr3 expression in mice.** *A,* representative immunostainings of Fpr3 expression in leukocytes and vomeronasal cells of C57Bl/6NCrl mice (left) versus 129X1/Sv mice (right). Fpr3 is only detectable in cells from C57Bl/6NCrl mice. *Scale bars,* 10 µm. *B,* quantification of Fpr3 expression in leukocytes (upper panel) and vomeronasal cells (lower panel). Bar charts show average percentage of stained cells from at least three independent experiments. Numbers in parentheses denote positive versus total cells. *Error bars,* S.D.

To investigate this in more detail, additional immunostainings in leukocytes from C57Bl/6NCrl, 129X1/Sv, BALB/cJ, FVB/N, and NZB/Ola mice were performed. Fpr3 protein expression was observed in NZB/Ola and C57Bl/6NCrl mice. By contrast, BALB/cJ, FVB/N, and 129X1/Sv mice showed no Fpr3 expression. However, Fpr3 mRNA expression from the VNO by the production and detection of cDNA in all negatively tested strains was still detectable. Possibly, variations in the Fpr3 gene alter the Fpr3 protein structure and thus prevent its detection by the antibodies. Therefore, I amplified and sequenced Fpr3 from genomic DNA of C57Bl/6NCrl and 129X1/Sv mice and compared the results (Figure 27).

This analysis indeed revealed two distinct Fpr3 variants: the Fpr3 sequence from C57Bl/6NCrl mice perfectly matched the annotated NCBI reference sequence NM_008042.2, whereas the Fpr3 sequence from 129X1/Sv mice showed a 12 nucleotide in-frame deletion (Figure 27A). This deletion comprised the nucleotides 424 to 435 of the coding region and resulted in the loss of alanine142, arginine143, asparagine144, and valine145 but left the open reading frame intact. For a clear discrimination, the version carrying the 12 nucleotide deletion was called Fpr3Δ424-435 and the full-length version was called Fpr3 wt.

Next, examinations on Fpr3 in BALB/cJ, FVB/N, and NZB/Ola mice revealed a clear correlation between specific gene variants and the presence or absence of Fpr3 antibody staining (Figure 27B). Fpr3Δ424-435 was present in the genome of 129X1/Sv, BALB/cJ, and
RESULTS

FVB/N mice that had no detectable Fpr3 protein whereas C57Bl/6NCrl and NZB/Ola, in which Fpr3 protein was readily detectable, carried the Fpr3\_wt gene. To obtain a more comprehensive view of the distribution of both Fpr3 variants in different inbred strains, the Mouse Genomes Project Database was consulted. The analyses revealed the occurrence of Fpr3\_wt in C57, C58, I, KK NOD, NZB, NZW, and ST mice, whereas 129S, 129P, AKR, A, BALB, BUB, C3H, CBA, DBA, FVB, LP, NZO, RF, and SEA all carried the Fpr3Δ424-435 variant.

Figure 27. Sequence and distribution of the 12 nucleotide in-frame deletion in Fpr3Δ424-435. A, genotyping of Fpr3 in 129X1/Sv and C57Bl/6NCrl mice revealed two receptor variants Fpr3\_wt and Fpr3Δ424-435. A 12 nucleotide in-frame deletion from base pair 424 to 435 was observed in 129X1/Sv mice resulting in a loss of an Alanine, Arginine, Asparagine, and Valine at the end of the second intracellular loop. Identical results were obtained from three individuals of each strain. B, distribution of both Fpr3 gene variants in different laboratory mouse strains. Gray shading denotes the genomically encoded variants. Bold letters annotate house sequenced strains. All other data were obtained from the Mouse Genome Project (https://www.sanger.ac.uk; release REL-1505). C, distribution of both Fpr3 gene variants in wild-derived mouse strains from different subspecies and geographical origins. Mus musculus castaneus was from Thailand (CAST/EiJ). M. m. domesticus were from Germany (GER/DT), France (FRA/DT), the United States of America (LEWES/EiJ, WSB/EiJ), and Switzerland (ZALENDE/EiJ). M. m. musculus were from Kazakhstan (KAZ/DT) and Czech Republic (CZE/DT, PWK/PhJ), M. m. spreitus was from Spain (SPRET/EiJ).
Because of this high frequency of both variants in laboratory mice, I asked about the possible origin of the two Fpr3 variants. The development of a receptor variant could have emerged under natural conditions or may have been a consequence of breeding conditions. Therefore, I was interested in determining whether both variants could be found in wild mouse strains and thus looked at the frequency of both Fpr3 variants in wild-derived animals. Most laboratory mouse strains are crossbreedings from three Mus musculus subspecies: M. m. castaneus, M. m. domesticus, and M. m. musculus. Hence, their genomes depict mosaics of the genomes of these three ancestor strains. To elucidate which of these ancestors carried the \(Fpr3\Delta_{424-435}\) variant, the Fpr3 sequence of wild-derived Mus musculus strains was investigated (Figure 27C). The genomes of wild-derived mouse strains, unlike that of laboratory inbred strains, mainly mimic the genome of only one ancestor strain.

My panel of six genomic DNAs covered samples of M. m. castaneus, M. m. domesticus, M. m. musculus, and an additional M. m. spretus sample. The samples were collected from different locations on the three continents North America, Europe, and Asia. CAST/EiJ was the only representative of M. m. castaneus originated in Thailand. GER/DT and FRA/DT representing M. m. domesticus were from Germany and France, respectively. The wild-derived strains representing M. m. musculus, KAZ/DT and CZE/DT, were from Kazakhstan and the Czech Republic. SPRET/EiJ, representing M. m. spretus, originated in Spain. The analysis of these DNA samples by direct sequencing of PCR products showed that they all carried \(Fpr3_{wt}\). Additional data mining in the Mouse Genomes Project Database indicated three M. m. domesticus lines, LEWES/EiJ, WSB/EiJ, and ZALENDE/EiJ from the United States of America and Switzerland, respectively, that carried the \(Fpr3\Delta_{424-435}\) variant. Thus, \(Fpr3\Delta_{424-435}\) was most likely introduced into inbred mouse lines through breeding with M. m. domesticus mice that accidentally carried \(Fpr3\Delta_{424-435}\).

3.3.2 Loss of Fpr3\(\Delta_{424-435}\) Function Due to Diminished Receptor Expression

The deletion in \(Fpr3\Delta_{424-435}\) leads to a loss of only four amino acids in the second intracellular loop but leaves the open reading frame intact (see chapter 3.3.1). The presence of \(Fpr3\) mRNA in the absence of a detectable protein strongly argues for structural alterations in \(Fpr3\Delta_{424-435}\). I thus hypothesized that these alterations may affect the receptor function. To test this, I used an established in vitro calcium imaging assay (Bu\{e\} et al., 2012) (Figure 28).

First calcium imaging in HEK cells transiently transfected with either \(Fpr3\Delta_{424-435}\) or \(Fpr3_{wt}\) were performed and calcium responses to 30 μM W-peptide were recorded. This substance was chosen for initial comparative functional experiments on Fpr3 because it is a
RESULTS

well-characterized Fpr agonist (Buße et al., 2012; Buße et al., 2015). 37% (177/475) of Fpr3<sub>wt</sub> transfected cells were robustly activated by W-peptide. However, none of the analyzed 686 cells transfected with Fpr3<sub>Δ424-435</sub> responded to this stimulus (Figure 28). This dramatic result was unexpected as the deletion left the open reading frame intact.

Figure 28. Fpr3<sub>Δ424-435</sub> does not respond to the synthetic Fpr3 agonist W-peptide. Single cell calcium imaging of HEK293T cells transfected with Fpr3<sub>Δ424-435</sub> or Fpr3<sub>wt</sub> upon W-peptide stimulation. Each trace represents an individual cell. Left: Fpr3<sub>Δ424-435</sub> transfected cells. None of 686 cells responded to 30 µM W-peptide. Right: Fpr3<sub>wt</sub> transfected cells. 177 of 475 cells (red) responded to 30 µM W-peptide. Buffer was used to exclude mechanical activation; 30 µM ATP that activates endogenous receptors was used as positive control for cell viability. Scale bars, vertical 0.5 340 nm/380 nm, horizontal 10 s.

I wondered whether this lack of function for Fpr3<sub>Δ424-435</sub> was specific for W-peptide, or if it also occurred with other ligands that activated Fpr3. To answer this question, I tested three bacterial signal peptide fragments – Salmonella-SP24, Psychromonas-SP6, and Hydrogenobacter-SP16 – that were recently identified as naturally occurring Fpr3 activators (Buße et al., 2015) and the synthetic M-peptide, which exhibits high sequence divergence to W-peptide (Figure 29). Fpr3<sub>Δ424-435</sub> transfected cells were neither activated by M-peptide nor any of the three sequence divergent bacterial signal peptides. By contrast, 26.0% ± 4.3% Fpr3<sub>wt</sub> transfected cells were activated by M-peptide and 30.0% ± 1.5%, 27.6% ± 3.8%, and 9.8% ± 2.4% of the cells were activated by the bacterial signal peptides, respectively. Thus, I concluded that the lack of Fpr3<sub>Δ424-435</sub> function was not dependent on the substance, but rather constitutes a general effect.
3.3.3 Lack of Fpr3Δ424-435 Expression in HEK Cells

The absence of specific Fpr3 staining in VNO cells and leukocytes from mice carrying the Fpr3Δ424-435 gene may have been caused by diminished or no expression of the Fpr3 protein. Therefore, the degree of protein expression by both Fpr3 variants was assessed by ECL1 and ECL2 immunostaining on HEK cells. Representative immunohistochemistry images showed clear staining for Fpr3wt with both antibodies (Figure 30). Supporting the results obtained in vomeronasal and immune cells, Fpr3Δ424-435 was not detected with either antibody. ECL2 stained 11.2% ± 1.7% of the Fpr3wt and 0.3% ± 0.2% of the Fpr3Δ424-435 transfected cells, whereas ECL1 stained 11.6% ± 2.7% and 0.4% ± 0.5%, respectively.

Figure 30. Expression of Fpr3Δ424-435 and Fpr3wt in HEK293T cells. Immunostainings of HEK293T cells expressing Fpr3wt (left) or Fpr3Δ424-435 (middle) and their quantification (right). Only Fpr3wt was detectable. The bar chart shows the average percentage of stained cells from three independent experiments. Numbers in parentheses denote positive versus total cells. Error bars, S.D. Scale bar, 20 µm.

The lack of specific Fpr3Δ424-435 staining observed in the overexpressing system closely resembled the lack of Fpr3Δ424-435 protein in VSNs and leukocytes (see chapter 3.3.1). The lack of staining by two independent antibodies that recognize different parts of the receptor
strongly suggests degradation of the Fpr3Δ424-435 variant in HEK cells. However, technical issues could also produce such results. To distinguish between these possibilities, I subcloned Fpr3 into a novel vector to create an Fpr3Δ424-435 fusion protein exhibiting specific N- and C-terminal epitopes. This strategy permitted independent detection of the receptor sites using two different antibodies specific for these epitopes. N-terminal rhodopsin-epitope (Rho) and C-terminal herpes simplex virus-epitope (HSV) tags were attached to Fpr3Δ424-435 and named the Rho-Fpr3Δ424-435-HSV fusion protein. Three independent copies were produced, sequenced, and tested to exclude any possible corruption in the vector (Figure 31).

The N-terminal Rho-tag showed pronounced staining of the transiently transfected cells. Rho-Fpr3wt stained 33.71% ± 5.79% of the cells, and Rho-Fpr3Δ424-435-HSV stained 9.65% ± 2.54% of the cells, representing a three-fold reduction of expression. The extent of receptor expression was examined with the C-terminal HSV-tag. The positive control was T2R16-HSV transfected cells, in which 30.23% ± 2.67% of the cells were stained. Rho-Fpr3Δ424-435-HSV showed complete loss of staining with the HSV antibody for all three of its copies.

![Image of immunostainings](image_url)

**Figure 31. Fpr3Δ424-435 exhibits truncated expression in HEK293T cells.** Immunostainings of HEK293T cells transfected with an independent plasmid copy of Rho-Fpr3Δ424-435-HSV that is a fusion protein of Fpr3Δ424-435 with an N-terminal Rhodopsin-epitope (Rho) and a C-terminal herpes simplex virus-epitope (HSV). Left: quantification of stainings with an anti Rho antibody. Right: quantification of stainings with an anti HSV antibody. Bars show average percentage of stained cells from three independent experiments. As controls...
Rho-Fpr3<sub>wt</sub>, a fusion protein of Fpr3<sub>wt</sub> with an N-terminal Rho-epitope, and T2R16-HSV, a fusion protein of T2R16 with a C-terminal HSV-epitope, were used. Error bars, S.D.

Taken together, these experiments strongly argue that structural alterations in Fpr3<sub>Δ424-435</sub> resulted in production of an unstable protein in HEK cells, which was C-terminally truncated and subsequently degraded. This mechanism is likely to be responsible for degradation of Fpr3<sub>Δ424-435</sub> in mouse VSNs and leukocytes.

### 3.3.4 Fpr3<sub>Δ424-435</sub> is Non-Functional in the Vomeronasal Organ

My previous findings showed strain-specific Fpr3 expression in two variants. The variant functional in HEK cells was called Fpr3<sub>wt</sub>, and the non-functional variant was called Fpr3<sub>Δ424-435</sub>. I was interested in determining whether these findings also affected the responses of VNO cells to Fpr3 agonists.

If Fpr3<sub>Δ424-435</sub> was non-functional in vivo, it should have affected the animal response and would therefore have influenced their infection susceptibility and all other potential functions and behaviors related to Fpr3. To test the functionality of both Fpr3 variants in vivo, calcium imaging experiments on whole-mount preparations of the sensory side of the VNOs from C57Bl/6NCrl and 129X1/Sv mice were performed with the potent Fpr3 activator Salmonella-SP24 in cooperation with Dr. Andreas Schmid (Figure 32). Dendritic endings on the sensory side contact the outside world and express different receptors, including Fprs (Dietschi et al., 2013). Based on my previous results I hypothesized that signals would only be detectable in C57Bl/6NCrl mice expressing Fpr3<sub>wt</sub>, whereas no signals would be detected in 129X1/Sv mice encoding Fpr3<sub>Δ424-435</sub>.

First, the VNOs of C57Bl/6NCrl mice were tested with three consecutive applications of 1 µM Salmonella-SP24. Several dendritic endings were robustly activated with each of the three signal peptide fragment applications (Figure 32A). In total, an area of ~300,000 µm<sup>2</sup> was analyzed in seven independent experiments. Of approximately 34,000 dendritic endings found in this area, 41 responded to repeated applications of the ligand (Figure 32B). A total of 0.12% ± 0.04% cells responded, which corresponded well with the immunostaining results (see chapter 3.2.1).

Subsequently, VNOs of 129X1/Sv mice expressing Fpr3<sub>Δ424-435</sub> were tested. A total area of ~100,000 µm<sup>2</sup> was examined in four independent experiments (Figure 32B). None of the approximately 12,000 dendritic endings in 129X1/Sv mice responded to any of the repeated applications of 1 µM Salmonella-SP24. These data show that VSNs in C57Bl/6NCrl mice,
expressing the functional Fpr3\textsubscript{wt}, can be activated by an Fpr3 activator, whereas cells in 129X1/Sv mice cannot. Taken together, these results demonstrate the novel discovery of two strain-specific Fpr3 variants that are functionally distinct.

Figure 32. Fpr3 agonists activate dendritic endings of VSNs. Calcium imaging on individual dendritic knobs of a whole mount preparation of VNOs of C57Bl/6NCrl and 129X1/Sv mice upon stimulation with 1 µM Salmonella-SP24. A, representative calcium traces of responding dendritic knobs. Each trace represents an individual knob of a C57Bl/6NCrl mouse that responds to each of three stimulations with the ligand, indicated by the arrows. Similar results were achieved over seven experiments. B, quantification of all measured dendritic knobs of both mouse strains. Bars represent the percentage of responding knobs upon stimulation. C, view on a section of the sensory side of the VNO. Dendritic knobs are depicted in green. Scale bar, 20 µm. The results were kindly provided by Dr. Andreas Schmid, Department of Physiology, Saarland University. Subfigure C was adapted and modified from Oboti et al., 2015.

3.4 Comparative Characterization of Murine and Human Fpr3 Function

In this study, Fpr3 was expressed in the VNO and the immune system of mice (see chapters 3.2.1 and 3.2.2). This expression pattern suggests an immune cell function for murine Fpr3 and argues for a close relationship with immune Fprs. Mouse Fpr1 has been suggested to be a human FPRI orthologue, whereas murine Fpr2 has been suggested to be a human FPR2 orthologue (Migeotte et al., 2006; Önheim et al., 2008; Dahlgren et al., 2016). Hence, I hypothesized that Fpr3 of both species could occupy the same functional niche. However, the evolutionary relationship between murine and human Fpr3 has not been clarified.
3.4.1 Orthology between Murine and Human Fpr3 is not Assessable by Sequence Comparison

The amino acid sequence of murine Fpr3 was compared to those of all human FPRs to investigate the evolutionary relationship between murine and human Fpr3 (Figure 33A). A sequence alignment analysis revealed only modest similarity between Fpr3 of both species. Murine Fpr3 was 82% similar with human FPR2, followed by 74% with human FPR3, and 72% with human FPR1. This result resembled the receptor similarities of all murine and human immune receptors in the dendrogram (Figure 33B). Murine Fpr3 was on the same branch with murine Fpr2 and separated from murine Fpr1 and human FPR1, which aligned on the second branch slightly nearer to human FPR2 than to human FPR3. Meta-analyses comprised of 12 orthology prediction tools (see chapter 2.8.2.2) revealed that the relationship between mouse and human Fpr3 was listed by only three of the tools, such as Compara, HGNC, and Panther, whereas Fpr1 and Fpr2 were annotated as human counterparts of FPR1 and FPR2 by 11 and nine consulted tools, respectively (Figure 33C). The other nine tools, such as HomoloGene, InParanoid, Isobase, OMA Browser, OrthoDB, OrthoMCL, PhylomeDB, Roundup, and TreeFam, did not list mouse and human Fpr3 as orthologues. These results suggest that the orthology between murine and human Fpr3 cannot be determined only by a sequence comparison. Thus, I next compared the functional properties of the two receptors.

Figure 33. Sequence relationship between murine Fpr3 and human Fprs. A. Comparison of amino acid sequence similarities between mouse Fpr3 and all three human Fprs. B. Phylogenetic relationship between all mouse and human Fprs. C. Integrative meta-analyses of the orthology of murine and human Fprs using the integrative search algorithm of DIOPT that combines orthology search results from 12 renowned ortholog prediction tools. The Meta-Score represents the number of individual tools listing murine and human Fprs as orthologs. Orthology between Fpr3 of mouse and human is only listed by Compara, HGNC, and Panther. hFPR = human Fpr; mFpr = murine Fpr.

3.4.2 Murine and Human Fpr3 Show Similar Functional Properties

To compare the function of murine and human Fpr3 to each other and in the context of the Fpr families, I first studied the concentration dependent responses of human FPR1, FPR2, and
FPR3 with those of murine Fpr1, Fpr2, and Fpr3 to the synthetic compounds W-peptide (WKYMVm-NH$_2$) and L-M-peptide (MMHWAM-NH$_2$), and the bacterially derived signal peptide *Psychromonas*-SP6 (f-MLFYFS) (Figure 34). These peptides show a considerable structural variability because their amino acid composition differed in all positions. The closest resemblance in their structure is that they all contain a methionine with a chemical modification. But even these methionines are chemically divergent: W-peptide ends with an amidated methionine in D-conformation (m-NH$_2$), whereas the amidated methionine at the end of L-M-peptide has an L-conformation (M-NH$_2$), and the signal peptide *Psychromonas*-SP6 starts with a formylated L-methionine (f-M).

Concentrations-response curves revealed clear similarities in the responses of specific mouse and human FPR receptor pairs to the selected test substances (Figure 34). Murine and human Fpr1 showed identical agonist preferences. Both receptors preferred the bacterial signal peptide *Psychromonas*-SP6, over W-peptide and L-M-peptide. Their half maximal activation values (EC$_{50}$) for the different stimuli were also closely related. For *Psychromonas*-SP6 the EC$_{50}$ were 0.15 nM and 0.048 nM respectively, for W-peptide 2.2 nM and 0.99 nM and for L-M-peptide 360 nM and 89.6 nM. The high similarity in the responses of murine and human Fpr1 to all three stimuli fits well to the concept of an identical function for both receptors in both species. Murine and human Fpr2 also displayed clear similarities. Both receptors showed similar EC$_{50}$ values for W-peptide and L-M-peptide and preferred W-peptide over the two other agonists (Figure 34). Only their EC$_{50}$ for L-M-peptide differed by approximately 50-fold. Interestingly, the responses of murine and human Fpr3 also correlated (Figure 34). First, I observed that both receptors were drastically less sensitive than Fpr1 and Fpr2 and usually responded in the high nanomolar to micromolar range. The W-peptide responses are a prime example for this behavior. The EC$_{50}$ of murine Fpr1, Fpr2 and human FPR1, FPR2 were 0.99 ± 0.23 nM, 0.36 ± 0.24 nM, 2.20 ± 2.12 nM, and 0.26 ± 0.19 nM, respectively, whereas the corresponding values of murine Fpr3 and human FPR3 were 676 ± 174 nM and 6117 ± 618 nM, respectively. A general lower sensitivity of murine and human Fpr3 was also visible for L-M-peptide and *Psychromonas*-SP6. Moreover, the maximal signal amplitudes of Fpr3 of both species to all tested stimuli were clearly reduced in comparison to the responses of Fpr1 and Fpr2 (Figure 34). These observations argue for functional similarities between murine Fpr3 and human FPR3.
**RESULTS**

Figure 34. **Murine and human Fpr3 show related agonist responses.** Concentration-responses of HEK293T cells expressing mouse (lower panel) and human (upper panel) Fpr1, Fpr2, and Fpr3 upon stimulation with four sequence divergent Fpr activators. EC50 values from all six receptors for the tested peptides are listed under the concentration-response curves. The number of experiments (n) is indicated above the curves. hFPR = human Fpr; mFpr = murine Fpr. Error bars, S.D.

### 3.4.3 Murine and Human Fpr3 are More Selective than Fpr1 and Fpr2

To further determine the degree of similarity between the agonist spectra of mouse and human Fpr3, I next compared their responses to a larger set of stimuli (Figure 35A). It is well-established that Fprs can respond to formylated bacterial and mitochondrial peptides with extraordinary sensitivity, which suggests these compounds being the prime activators of Fprs (Schiffmann et al., 1975; Le et al., 2001a; Tiffany et al., 2001; Harada et al., 2004; Rabiet et al., 2005; He et al., 2013). Therefore, I focused on these agonist families. A panel of 23 compounds that varied significantly in structure, sequence and length was tested (Figure 35B). It contained 12 different formylated mitochondrial peptides (three of human and nine of murine origin), seven formylated bacterial peptides, and four other typical peptide activators of FPRs. All compounds were first tested at 10 µM to 30 µM on the six receptors murine Fpr1, Fpr2, Fpr3 and human FPR1, FPR2, FPR3. The results show that Fpr1 and Fpr2 of mouse and human are capable of detecting a broad variety of structurally divergent peptides of mitochondrial or bacterial origin, while murine and human Fpr3 are far more selective.

Clear parallels between responses of distinct mouse and human receptor pairs were revealed (Figure 35A). Murine and human Fpr1 showed the most homogeneous activation pattern. Both receptors were strongly activated by all 23 test compounds and their response
amplitudes were always similar to those of W-peptide. Murine and human Fpr2 also displayed related responses. Both receptors were activated by 22 of the 23 substances. In contrast to the homogeneous signal amplitudes of Fpr1 the responses of Fpr2 to a few compounds were clearly diminished. This effect was most pronounced for mATP6 and mND4L. For human FPR2 the signals to mATP6 and mND4L were reduced by 79% and 81%, respectively. Murine Fpr2 showed a similar reduction to mND4L and no signal to mATP6. In addition, mouse Fpr2 also showed responses that were more than 60% reduced to mCytb, mND6, mND5, mND3, mND2, and hND4 which were not visible for human FPR2.

Interestingly, clear similarities between murine and human Fpr3 were also observable. Both receptors showed a specific response pattern that was clearly distinguishable from that of Fpr1 and Fpr2 of both species. Both receptors responded to a far smaller set of compounds than their family members. Human FPR3 was only activated by 14 of the 23 tested substances – Staphylococcus-SP22, Staphylococcus-SP22-FuLe, Salmonella-SP24, Psychromonas-SP6, Hydrogenobacter-SP16, hND6, D-M-peptide, W-peptide, f-MIVILY, mND2, mND4, mND4L, mCOIII, and Ac2-26. Murine Fpr3 was even only activated by nine substances – Staphylococcus-SP22, Staphylococcus-SP22-FuLe, Salmonella-SP24, Psychromonas-SP6, Hydrogenobacter-SP16, hND6, M-peptide, W-peptide and f-MIVILY. The observation that human FPR3 was only activated by 14 compounds and murine Fpr3 even responded to only nine test substances argues for a higher selectivity of both receptors than that of Fpr1 and Fpr2. It is to mention that all activators of murine Fpr3 were also activators of human FPR3 what results in an overlap of the receptors’ ligand spectra by at least 64%. The fact that all mouse Fpr3 agonists overlapped to 100% with those of human FPR3 strongly support the hypothesis that murine and human Fpr3 fulfill similar roles in the immune systems of mouse and human. However, the fact that human FPR3 is activated by five additional test compounds (Ac2-26, mND2, mND4, mND4L, and mCOIII) illustrates species-specific adaptations in the sensitivity and selectivity of both receptors.
RESULTS

Figure 35. Mouse and human Fpr3 display similar agonist selectivity. A. Comparison of the calcium responses of HEK293T cells transfected with either Fpr1, Fpr2, or Fpr3 of mouse or human to high agonist concentrations. All compounds were applied at 10 µM to 30 µM. Buffer application was used as negative control to render mechanical stimulation visible. To account for receptor specific variations in the maximal obtainable signal amplitude each response was normalized to the response to 30 µM W-peptide, a pan agonist for murine and human Fpr1, Fpr2 and Fpr3. Bars denote mean signals from three to eight independent experiments, carried out as duplicates. Error bars, S.D.

3.4.4 Specification of Fpr3 Function in Mouse and Human

To assess the amount of species-specific alterations in more detail, I next compared the concentration responses of both receptors to four common activators: the human mitochondrial peptide hND6, the bacterial signal peptide Psychromonas-SP6 and to the D- and L-stereoisomers of the synthetic M-peptide (Figure 36). The responses of murine and human Fpr3 to hND6 and L-M-peptide were quite similar, whereas the receptors displayed clear differences in the responses to D-M-peptide and Psychromonas-SP6. The EC50 of Fpr3 for D-M-peptide and Psychromonas-SP6 were 933 ± 432 nM and 347 ± 49 nM, respectively. Human FPR3 showed an EC50 value of more than 10,000 nM for both substances. Thus,
murine Fpr3 is at least 10-fold more sensitive for D-M-peptide and even 28-fold more sensitive for *Psychromonas*-SP6. I also noticed that the maximal signal amplitude of murine Fpr3 to L-M-peptide and hND6 were approximately 50% smaller than those to D-M-peptide and *Psychromonas*-SP6, although the receptor signals clearly reached saturation for all four compounds. This provided the possibility for partial activation of murine Fpr3 through both compounds.

![Graphs showing concentration-response of murine and human Fpr3](image)

**Figure 36. Mouse and human Fpr3 differ in their agonist sensitivity.** Concentration-responses of murine and human Fpr3 upon activation by four structurally divergent peptides reveal a differential sensitivity. Number of experiments (n) is indicated in parentheses. hFPR = human Fpr; mFpr = murine Fpr. Error bars, S.D.

To examine whether this was true or if the differences in the signal amplitude were caused by non-saturated responses, I next compared the signal amplitudes to selected agonists at 30 μM and 60 μM concentrations (Figure 37). If the responses were not in saturation one would expect an increase of the signal amplitude at the higher concentration. In case that the compounds were partial agonists, higher concentrations should not have altered the signal size. For these tests four common activators of Fpr3 of both species (hND6, *Staphylococcus*-SP22, *Psychromonas*-SP6, and *Hydrogenobacter*-SP16), two peptides that were selective activators of human FPR3 (mND2 and mND4L), and two controls that did not activate murine or human Fpr3 (mATP6 and *Clostridium*-SP13) were chosen based on the results obtained in previous experiments (Figure 34). No significant differences in the signal amplitudes of murine and human Fpr3 to 30 μM and 60 μM stimulus concentrations were observed. hND6, *Staphylococcus*-SP22, *Psychromonas*-SP6, and *Hydrogenobacter*-SP16 activated murine Fpr3 at both concentrations but showed no increase in signal size (Figure 37). Responses of human FPR3 to hND6, mND2, mND4L, *Psychromonas*-SP6, *Hydrogenobacter*-SP16, and *Staphylococcus*-SP22 also induced no significant alterations. Additionally, all tested compounds that did not activate one of the receptors at 30 μM also
RESULTS

evoked no responses at 60 µM. This demonstrates that the receptor responses were in saturation at 30 µM.

Next, I obtained clear evidence for partial agonists of murine Fpr3. Although the results indicate sufficient concentrations to monitor the maximal signal amplitude, some of the signals were much smaller than those obtained for W-peptide. For murine Fpr3 only the signal size of *Psychromonas*-SP6 was comparable to that of W-peptide. The signal amplitudes of hND6, *Hydrogenobacter*-SP16, and *Staphylococcus*-SP22 were reduced by 74%, 65%, and 61% respectively, providing clear evidence that these three stimuli are indeed partial agonists of murine Fpr3. By contrast, nearly all of the identified agonists for human FPR3 were full activators. The signals of this receptor to hND6, mND2, mND4L, *Hydrogenobacter*-SP16, and *Staphylococcus*-SP22 were similar to those obtained with W-peptide (Figure 37). Only the response to *Psychromonas*-SP6 was reduced by 79%. Thus, it seemed that Fpr3 was more selective in terms of signal amplitude.

Figure 37. Calcium responses of murine and human Fpr3 saturate at 30 µM ligand concentration. Calcium responses of cells transfected with murine (light blue, upper panel) or human (dark blue, lower panel) Fpr3 upon stimulation with 30 µM (filled bars) and 60 µM (shaded bars) of the indicated Fpr3 agonist. All eight activators showed no significant differences between both concentrations, demonstrating that signal saturation was reached at 30 µM. Bars denote mean signal amplitudes from three independent experiments, carried out as duplicates. Number of experiments (n) is indicated in parentheses. hFPR = human Fpr; mFpr = murine Fpr. Error bars, S.D.

3.4.5 Ligand Preferences of Murine and Human Fpr3 Differ Partially

To assess to what extent variations in the ligand structure affect the responses of murine and human Fpr3, I next tested both receptors with a number of closely related chemical derivatives of the same agonist. Bernd Bufe and colleagues recently reported varying importance of individual amino acid residues of W-peptide for the interaction with murine Fpr3 (Bufe et al., 2012) and human FPR3 (Bufe et al., 2015). W-peptide is a common activator of mouse and human Fpr3 that robustly activates both receptors (Figure 34). Thus, I reasoned that structural derivatives of W-peptide may be well-suited to reveal differences in the agonist preferences.
RESULTS

Previous studies already demonstrated the exclusive importance of the amidated methionine at the C-terminus of W-peptide and the three following residues for the interaction between W-peptide and the receptor (Bufl et al., 2012, Bufl et al., 2015). Therefore, I focused on systematic tests of these four residues with a panel of 24 test compounds (Figure 38). In each peptide an individual amino acid of W-peptide was replaced by a non-polar, polar, aromatic or charged substitution. My results showed that alterations of the C-terminal D-methionine (m) affected the responses of murine and human Fpr3 in a similar manner (Figure 38). At this position, charged or polar residues, such as glutamate (e), lysine (k) or glutamine (q) totally abolished the responses of both receptors. Replacement by hydrophobic residues, such as alanine (a), cysteine (c), isoleucine (i), and ornithine (o) or amino acids in L-conformation led to drastically reduced signals. Only the substitution of D-methionine by homocysteine (hcy) or isoleucine (i) were relatively well-tolerated. Thus, the methionine was of equal importance for the ligand recognition of both receptors. Alterations at the second last residue showed little effects on the receptor responses (Figure 38), suggesting low importance for the interaction between W-peptide and the receptors at this position. Replacement of the third last residue by other amino acids was well-tolerated by murine Fpr3, whereas all responses of human FPR3 were diminished. The substitution of the hydrophobic methionine (M) by charged glutamate (E) led to the strongest difference. It totally abolished the response of human FPR3, whereas the response of murine Fpr3 was only diminished by 43% (Figure 38). The most pronounced differences between murine Fpr3 and human FPR3 were seen for substitutions at the fourth last position (Figure 38). Interestingly most replacements of this residue by another amino acid were well-tolerated by murine Fpr3. In sharp contrast, nearly all substitutions totally abolished the response of human FPR3. This receptor tolerated only the relatively conserved exchange of tryptophan (W) by phenylalanine (F), both of which are aromatic amino acids. However, even in this case a marked reduction in the signal amplitude was recognized. This indicates differences in the response of murine and human Fpr3 to agonists are primarily that are caused by variable preference of both receptors of the fourth residue after the chemical modification. Furthermore, both receptors show comparable signal amplitudes to the majority of the W-peptide derivatives. In summary, the test with W-peptide derivatives revealed definite similarities in the structural preferences of murine and human Fpr3.
RESULTS

Figure 38. **The ligand recognition of murine and human Fpr3 partially differs.** Calcium responses of HEK293T cells transiently transfected with either Fpr3 of mouse (light blue, upper panel) or human (dark blue, lower panel) to W-peptide derivatives (10 µM) with systematically exchanged amino acid residues at position 1, 2, 3, and 4 (counted from the amidated C-terminus). Buffer application (black bar and dotted line) was used as negative control. To account for receptor specific variations in the maximal obtainable signal amplitude the responses were normalized to 30 µM W-peptide (gray bar and dotted line). Bars denote signal amplitudes of responding cells from four independent experiments. The lead structure of W-peptide is shown in black letters. Modifications in the peptide structure are labeled in red. Peptide sequences are shown in one-letter amino acid code. L-isomers are given in capital letters, whereas D-isomers are displayed in lowercase letters. -NH₂ = amidated C-terminus. hFPR = human Fpr; mFpr = murine Fpr. Error bars, S.D.
4 DISCUSSION

This thesis provides new insight into the expression and function of murine Fpr3 in the vomeronasal and immune systems and reports the existence of natural knockout strains for this receptor.

Two murine Fpr3 variants, $Fpr3_{\text{wt}}$ and $Fpr3\Delta_{424-435}$, which differed by 12 nucleotides were discovered during this study. $Fpr3_{\text{wt}}$ constituted the annotated receptor, whereas $Fpr3\Delta_{424-435}$ encoded an in-frame deletion from nucleotides 424–435. Immunocytochemistry revealed that the deletion left the open reading frame intact, but the mouse strains encoding $Fpr3\Delta_{424-435}$ did not express the Fpr3 protein. In vitro calcium imaging and immunofluorescence analyses demonstrated that the lack of four amino acids lead to an unstable, truncated, and non-functional receptor protein. Moreover, comprehensive genotyping analyses and a database search revealed at least 13 mouse strains expressing $Fpr3_{\text{wt}}$ and at least 19 other strains encoding $Fpr3\Delta_{424-435}$; thus establishing various natural Fpr3 knockout mouse strains. Sequencing and genomic haplotype analyses attributed the origin of $Fpr3\Delta_{424-435}$ to the subspecies Mus musculus domesticus. The discovery of a multitude of natural Fpr3 knockout mouse strains will be valuable to study murine Fpr3 function in the context of various genetic backgrounds.

Murine Fpr3 showed significant sequence overlap with human FPR3. Both receptors have been reported to detect bacterial peptides. Thus, they are assumed to fulfill related biological roles. However, the two receptors differed in their expression patterns, which challenge the concept of congruent function. The function and receptor expression of murine and human Fpr3 were examined in the vomeronasal organ and immune system to better understand their roles. In vitro calcium imaging experiments showed that the functional properties of the receptors were similar. Ligand screening revealed overlapping agonist response patterns in which the agonists were mainly of bacterial origin. Two anti-Fpr3 antibodies were generated and validated to analyze the occurrence of the murine Fpr3 protein and examine receptor expression. Immunocytochemistry combined with RT-PCR and in situ hybridization revealed murine Fpr3 expression in a subset of vomeronasal sensory neurons, mature neutrophil granulocytes, and bone marrow cells, whereas RT-PCR demonstrated the presence of human $FPR3$ RNA in neutrophil granulocytes. Moreover, expression of the murine Fpr3 protein was upregulated in immune cells upon stimulation with a bacterial endotoxin (lipopolysaccharide). Taken together, my results provide clear evidence for a common biological function of murine and human Fpr3 and support their role as bacterial sensors in immune defense.
4.1 Fpr3 of Mouse and Human are Functional Orthologs

Identification of human orthologs in model organisms, such as the house mouse *Mus musculus*, is of paramount importance for insight into human biology (Dolan et al., 2015). Orthologs are genes in different species that evolved from a common ancestral gene by speciation (Fitch, 1970; Fitch, 2000; Fang et al., 2010). Such genes can be identified by two criteria – a high sequence homology and the same biological function (Tatusov et al., 1997; Fang et al., 2010). I compared the sequence and function of murine and human Fpr3, to investigate to what degree both receptors fit these criteria.

4.1.1 Genetic Evidence for the Orthology between Fpr3 of Mouse and Human

Isabelle Migeotte and colleagues recently reported a shared gene locus with conserved synteny in the genomic arrangement for Fpr3 of both species, as described earlier (see chapter 1.3.4) (Migeotte et al., 2006). They illustrated that murine and human Fpr3 are located on chromosomal regions 17A3.2 and 19q13.3, respectively, where they are both the third protein coding gene within the Fpr gene cluster. However, due to the Fpr gene cluster expansion in rodents (Figure 5C), flanking regions of murine Fpr3 that comprise the pseudogenes ψFpr-rs2 (Fpr-rs8) and ψFpr-rs3 (Fpr-rs5) challenge the syntenic arrangement of murine and human Fpr3 (Gao et al., 1998; Wang and Ye, 2002). It is conceivable that all three of these genes evolved parallel to the human FPR3 gene. However, the nucleotide sequences of ψFpr-rs2 and ψFpr-rs3 are both more homologous to Fpr2 than to Fpr3 (Gao et al., 1998; Tiffany et al., 2011) which suggests evolution alongside this receptor. Furthermore, the two pseudogenes include premature stop codons (Migeotte et al., 2006) and thus do not have open reading frames. Hence, the Fpr3 gene encodes the only intact GPCR of these three genes. These observations consider Fpr3 to be the only mouse receptor qualified as ortholog of human FPR3.

To examine this hypothesis, I compared the amino acid sequences of murine and human Fpr3, and aligned them with those of Fpr1 and Fpr2 of both species (Figure 33A, B). Surprisingly, by sharing 82% similarity, mouse Fpr3 rather resembled human FPR2 than human FPR3 that was 74% similar with murine Fpr3. This finding is consistent with other studies (Takano et al., 1997; Gao et al., 1998; Rabiet et al., 2011) and challenges orthology between murine and human Fpr3.

In line with these observations, meta-analyses comprised of 12 orthology prediction tools listed orthology for murine and human Fpr3 in only three of these tools (Figure 33C), underpinning the notion that their orthology is unclear. However, a recent study that examined
the adaptive evolution of Fprs in mammals reported that Fpr3 evolved under completely different conditions than Fpr1 and Fpr2 (Muto et al., 2015). With examinations of site- and lineage-specific selection patterns, combined with 3D homology modeling analyses, the authors indicated strong positive selection for the Fpr1 and Fpr2 genes during evolution. By contrast, selective pressure in the Fpr3 lineage was more relaxed (Muto et al., 2015). This argues for common evolution of murine and human Fpr3 and provides a possible explanation for their orthology despite not sharing the highest sequence similarity amongst Fprs.

4.1.2 Fpr3 of Mouse and Human Share Functional Similarities

The same biological role is a criterion for orthology (Remm et al., 2001; Fang et al., 2010), as mentioned before (see chapter 0). Thus, I compared the function of murine and human Fpr3. Examining the biological role of Fpr3 has been difficult in the past due to the lack of identified activators. Recent studies identified several synthetic, bacterial, and endogenous host peptides as activators of mouse and human Fpr3 (Harada et al., 2004; Ernst et al., 2004; Migeotte et al., 2005; Bufe et al., 2012, Bufe et al., 2015). This enabled me to assess functional orthology between murine and human Fpr3.

In heterologous calcium imaging experiments both receptors responded to overlapping subsets of Fpr agonists from different origins. Murine and human Fpr3 responded to nine common ligands, thus overlapping by at least 64% (Figure 35). This is consistent with a study that compared the receptors’ responses to 21 bacterial signal peptide fragments (Bufe et al., 2015) and provides first evidence for a similar function of both receptors.

Five of the nine shared ligands for murine and human Fpr3 were bacterial signal peptides. By contrast, endogenous host stimuli, such as mitochondrial peptides, were no prominent agonists of murine and human Fpr3. Both receptors responded to only one of three human mitochondrial peptides. Murine Fpr3 even lacked activation through any tested mitochondrial peptide of the mouse. This finding is consistent with reports that described detection of bacterial peptides by murine and human Fpr3 (Betten et al., 2001; de Paulis et al., 2004; Bufe et al., 2015). These data suggest prime roles for murine and human Fpr3 in bacterial detection, while they are less important in endogenous host stimuli detection.

Response characteristics of Fpr3 differed drastically from those of Fpr1 and Fpr2 which are promiscuous receptors (Migeotte et al., 2006; Fu et al., 2006). In my screening, these receptors responded to nearly all of the 23 tested ligands (Figure 35). By contrast, Fpr3 of both species detected only a small sub-fraction of the tested ligands. Thus, Fpr3 was much more narrowly tuned than the other Fpr family members. Concentration-response curves
DISCUSSION

revealed also differing sensitivity and maximal signal amplitudes for Fpr3 towards Fpr1 and Fpr2 (Figure 34). To all tested substances – W-peptide, M-peptide, the bacterial signal peptide Psychromonas-SP6, and the mitochondrial peptide hND6 – Fpr3 exhibited ~3-fold to ~30-fold lower sensitivity than Fpr1 and Fpr2 of both species. Additionally, signal amplitudes of Fpr3 reached only ~20% to ~50% of those of Fpr1 and Fpr2. Drastically lower sensitivity of Fpr3 to bacterial signal peptides has been described before (Bufe et al., 2015). While Fpr1 and Fpr2 were activated by many substances in the nanomolar range, Fpr3 was activated only in the micromolar range. A more specialized ligand spectrum argues for a specific niche of Fpr3 within the murine and human Fpr families. This could characterize Fpr3 as a receptor providing information about high concentrations of external stimuli.

A speculated function of Fpr3 in the literature is that of a decoy receptor. These receptors bind ligands to inhibit binding to their destined receptor. Thereby, they exhibit minimal plasma membrane expression but undergo rapid constitutive recycling to bind extracellular ligands and internalize them for degradation. In this process they do not transduce signals (Rabiet et al., 2011). Several recent studies support the idea of Fpr3 being a decoy receptor, based on a relative insensitivity to common Fpr2 ligands and high basal levels of receptor phosphorylation and internalization (Migeotte et al., 2005; Rabiet et al., 2011; He et al., 2013; Dorward et al., 2015). These reports suggest regulation of the function of other formylated peptide receptors through Fpr3. However, Fpr3 showed sensitivity in the micromolar range to most tested ligands that is at least 1,000-fold lower than sensitivities of Fpr1 and Fpr2 in the nanomolar range (Figure 34). With such inferior sensitivity, Fpr3 could not hinder ligands from binding to Fpr1 or Fpr2. Thus, my data argue against decoy activity mediated by Fpr3.

4.1.3 Adaptations of Murine Fpr3 for a Function in Olfaction

Murine Fpr3 was initially discovered in the VNO (Liberles et al., 2009; Rivièrè et al., 2009), whereas no evidence exists for human FPR3 expression in the olfactory system (Liman and Innan, 2003; Zhang and Webb, 2003). Thus, it is likely that, despite functional orthology between murine and human Fpr3, murine Fpr3 developed specific adaptations to requirements in olfaction. To examine this, I compared the function of murine and human Fpr3 in more detail. Indeed, concentration-response curves supported the hypothesis of functional adaptations by revealing sensitivity differences between murine and human Fpr3 to two of four tested ligands (Figure 36). Thus, although both receptors detected the same ligands, they showed partial differing ligand tunings.
Investigations on responses of both receptors to single amino acid variations in W-peptide – a potent common ligand – were made to assess the degree of these subtle functional differences. Screening of 24 W-peptide derivatives (see chapter 2.1.1) revealed differing response patterns for murine and human Fpr3 (see chapter 3.4.5). Residue exchanges at the fourth last position of W-peptide showed the most distinct difference in the responses of the two receptors. The other three exchanged amino acid positions showed no or only minor differences between receptor responses (Figure 38). This finding indicates a possible adaptation of murine Fpr3 to a function in the VNO. However, it also shows that the functional differences between murine and human Fpr3 are very subtle. It is conceivable that these slight tuning differences indicate a trend in the evolution of both receptors that will become more pronounced in the future. Thus, murine Fpr3 function might adapt to requirements in olfaction, whereas human FPR3 might become more specialized for its function in the immune system.

4.2 Murine Fpr3 is Expressed in Multiple Tissues

Controversial reports about the expression pattern of murine Fpr3 exist, as described earlier (see chapter 1.3.3) (Gao et al., 1998; Lee et al., 2004; Southgate et al., 2008; Rivière et al., 2009; Chiu et al., 2013). To clarify if murine Fpr3 is expressed in immune cells, I examined the occurrence of receptor protein in different organs with immunocytochemistry techniques. To this end I generated and characterized two Fpr3-specific antibodies, ECL1 and ECL2 (see chapter 3.1).

4.2.1 Fpr3 Protein is Expressed in the Vomeronasal Organ

Specificity of an antibody is a general issue in immunocytochemistry. Thus, a number of methods were used to assure the quality of the newly generated antibodies. Epitope mapping (Figure 13) and heterologous immunocytochemistry experiments, in which murine Fpr3 was stained exclusively (Figure 14), provided substantial in vitro evidence for the specificity of both antibodies. Subsequent comparative tests identified ECL2 as the more sensitive antibody that also produced slightly less background (Figure 15). Thus, this antibody was primarily used for further experiments in native cells. The VNO provided perfect testing conditions to assess the quality of the antibody, because of consistent reports on Fpr3 expression in the literature (Liberles et al., 2009; Rivière et al., 2009) and a challenging amount of target cells (0.3% to 0.7%) (Rivière et al., 2009; Stempel et al., 2016). In agreement with previous reports
DISCUSSION

(Liberles et al., 2009; Rivière et al., 2009), Fpr3 was detected in a small subpopulation of ~0.3% VSNs with immunocytochemistry, RT-PCR, and in situ hybridization techniques (Figures 16, 17). This amount of cells is consistent with a study that recognized Fpr3 mRNA in 0.7% of the cells in VNO slices (Rivière et al., 2009). Verification of staining specificity was realized by successfully blocking the epitope specific binding site with the peptide that was used for antibody generation (Figure 17). These results confirmed the high quality of my used antibody for immunocytochemistry experiments in native cells.

Next, the molecular characteristics of the cells expressing Fpr3 protein in the VNO were examined (Figure 18). The studies that discovered Fpr3 in the VNO reported coexpression of the receptor in VSNs with Go, and a lack of coexpression with Gi2 (Liberles et al., 2009; Rivière et al., 2009), markers for VSNs of the basal and the apical expression zone, respectively (Halpern et al., 1995; Berghard and Buck, 1996; Herrada and Dulac, 1997; Matsunami and Buck, 1997). Furthermore, tests using a pan V2ra in situ probe detecting 17 of the altogether 122 type 2 vomeronasal receptors indicated a lack of Vmn2r expression in Fpr3-positive VSNs (Liberles et al., 2009). My findings, using colabeling immunocytochemistry techniques were fully consistent with these reports (Liberles et al., 2009; Rivière et al., 2009). The majority of Fpr3-positive cells colocalized with Go. Furthermore, neither significant coexpression of Fpr3 with Phosphodiesterase 4A (PDE4A), which labels Gi2-positive VSNs (Lau and Cherry, 2000; Leinders-Zufall et al., 2004), nor with type 2 vomeronasal receptors, were detected. These results confirmed the previous findings which showed that Fpr3 in the VNO is exclusively expressed in VSNs of the basal expression zone and does not coexpress with type 2 vomeronasal receptors on the protein level (Liberles et al., 2009; Rivière et al., 2009). Fpr3 was also colabeled with OMP, which is expressed in all mature VSNs, to examine if the dissociated cells expressing Fpr3 were VSNs (Margolis, 1982). Indeed, ~60% of the Fpr3-expressing cells in the dissociated cell preparation coexpressed Fpr3 with OMP (Figure 18) and thus were identified as VSNs. Surprisingly, another ~40% of the Fpr3-positive cells lacked colocalization with any VSN-specific marker and thus provide clear evidence for the existence of a second Fpr3-expressing cell type in my dissociated cell preparation.

Colocalization immunocytochemistry experiments with different immune cell markers were performed to examine the second cell type in my preparation (Figure 19). The lymphocyte antigen 6G (Ly6G) is only present in neutrophil granulocytes (Fleming et al., 1993; Lai et al., 1998), whereas the cluster of differentiation molecule (CD45R) can be found in all other immune cells (Ballas and Rasmussen, 1993; Rolink et al., 1996; Lai et al., 1998).
Colabeling ECL2 with Ly6G identified these cells as neutrophil granulocytes. Consistent with this finding, colabeling with CD45R was negative (Figure 19). Ly6G expression and the lack of CD45R expression argue that these cells were neutrophil granulocytes. Neutrophils are leukocytes that are released from the bone marrow into the peripheral blood after maturation (Bekkering and Torensm, 2013). Thus, they were likely introduced into my preparation through blood contamination.

4.2.2 Fpr3 Protein is Expressed in Immune Cells

Careful examination of Fpr3 expression in cells that were directly isolated from blood was performed to confirm the receptor’s occurrence in murine leukocytes. With both newly generated Fpr3 antibodies, ECL1 and ECL2, Fpr3 expression in a subpopulation of ~13% of the nucleated cells was detected (Figure 20). This amount of cells correlates well with the reported strain-dependent neutrophil granulocyte count of ~9% to ~18% in mouse blood (Gowen and Calhoun, 1943). Additional investigation on nucleus morphology of stained cells substantiated the evidence for Fpr3 expression in neutrophil granulocytes. Nuclei of Fpr3-expressing cells all had a structure including nuclear lobes and connecting segments, the typical shape of nuclei of mature neutrophil granulocytes (Campbell et al., 1995; Sanchez and Wangh, 1999; Carvalho et al., 2015); whereas all non-stained cells had other nucleus shapes (Figure 39). Fpr3 protein expression in neutrophil granulocytes was confirmed by subsequent colabeling of the receptor with the neutrophil marker Ly6G. Fpr3 expression in leukocytes was also proven on the RNA level with RT-PCR experiments (Figure 23). Interestingly, Fpr3 protein production increased after LPS exposure (Figure 25). Taken together, these results clearly demonstrate the presence of murine Fpr3 in neutrophil granulocytes.

Figure 39. Fpr3-positive leukocytes contain polymorphonuclear nuclei. Comparison of nuclear morphologies for Fpr3 positive and negative leukocytes. Fpr3 was stained with the antibody ECL1 (green), for nuclear staining Hoechst33342 (blue) was used. Fpr3-positive cell (upper left) showing a clearly multi-lobed nucleus, typical for neutrophil granulocytes (lower left). Fpr3-negative cell (upper right) showing a horseshoe-shaped nucleus, typical for monocytes (lower right). mFpr = murine Fpr. Scale bar, 5 µm.

Fpr3 expression in leukocytes has been discussed controversially in the past. One early report detected low amounts of Fpr3 RNA in murine leukocytes (Gao et al., 1998). However, several subsequent studies failed to confirm this result despite the use of sensitive techniques,
such as RT-PCR (Lee et al., 2004; Southgate et al., 2008; Rivière et al., 2009; Chiu et al., 2013). Detection of RNA from neutrophil granulocytes is much more difficult than from other leukocytes because RNA amounts in neutrophil granulocytes are 10-fold to 20-fold lower than in other leukocytes, such as monocytes (Cassatella, 1999). Consistent with the majority of reports, my attempts failed to detect Fpr3 from neutrophil granulocyte RNA without LPS stimulation (Figure 22). In immune cells expression of selected genes is elevated after contact with a pathogen (Guha and Mackman, 2001; Heumann and Roger, 2002). One important pathogen factor for this is LPS (Cui et al., 2002; Iribarren et al., 2003). In neutrophil granulocytes, LPS-induced expression for Fpr1 has been observed earlier (Mandal et al., 2005). Thus, it is conceivable that Fpr3 is also upregulated after LPS stimulation in this cell type. Utilizing this RNA elevation mechanism, I could now establish Fpr3 joining the ranks of upregulated genes upon LPS exposure in neutrophils (Figure 23). This finding resolves the controversy about Fpr3 expression in mouse leukocytes and strongly supports the involvement of Fpr3 in immune defense.

Of note, occurrence of murine and human Fpr3 in the same type of immune cells would provide further evidence for a similar biological function of both receptors in the immune systems of mouse and human. However, current literature gives no evidence for a common expression of murine and human Fpr3 in neutrophil granulocytes. RNA of human FPR3 has been detected in monocytes using northern blot analysis (Durstin et al., 1994) and in immature and mature dendritic cells via RT-PCR, immunocytochemistry, and internalization experiments (Yang et al., 2002; Migeotte et al., 2005). However, expression of human FPR3 in neutrophil granulocytes is poorly examined. To clarify FPR3 expression in human neutrophil granulocytes, I isolated neutrophils from peripheral blood, stimulated the cells with LPS, and extracted their total RNA. RT-PCR showed a band for human FPR3 with specific primers besides the already well-examined FPR1 and FPR2 (Figure 24). Consecutive sequencing confirmed the amplification products as human FPR1, FPR2, and FPR3. This pilot study shows that human FPR3 is likely expressed in human neutrophils.

### 4.3 Strain-Specific Fpr3 Variants

During the course of my studies, I made a puzzling observation: Fpr3 protein was not detectable by immunocytochemistry in dissociated VNO cells or immune cells of 129X1/Sv, BALB/cJ, and FVB/N mice but in C57Bl/6NCrl and NZB/Ola mice (Figure 26). However, the presence of Fpr3 mRNA in those cell types of all tested mouse strains was clearly proven.
DISCUSSION

(see chapter 3.3.1). Subsequent genotyping of the Fpr3 gene from Fpr3-positive and -negative strains revealed a clear correlation between antibody staining patterns and the presence or absence of a 12 nucleotide in-frame deletion in Fpr3 (Figure 27).

4.3.1 Two Functionally Distinct Fpr3 Variants Exist in Mice

The deletion results in a loss of four amino acids in the second intracellular loop. The Fpr3 variant comprising the deletion was called Fpr3Δ424-435, the variant without the deletion, Fpr3wt. Fpr3wt and Fpr3Δ424-435 were compared with calcium imaging to evaluate if the structural alteration in the Fpr3 variants would affect the receptor function (see chapter 3.3.2). The relatively small change of four amino acids at an intracellular site argues for modest effects on the receptor function. However, initial heterologous experiments in HEK cells revealed drastic functional differences between the two Fpr3 variants. Fpr3Δ424-435 transfected HEK cells responded to none of the applied potent Fpr3 activators, while those transfected with Fpr3wt clearly responded to all of them (Figures 28, 29). These results provide clear evidence for a complete loss of receptor function for the Fpr3Δ424-435 variant. A lack of receptor function should have been also observable in mice, whereby mice carrying Fpr3Δ424-435 should have displayed a lack of function and mice expressing Fpr3wt should have shown intact receptor function. To examine this hypothesis, the response of vomeronasal sensory neurons of C57Bl6/NCrl and 129X1/Sv mice was tested (Figure 32). When applying the bacterial signal peptide fragment Salmonella-SP24, a specific subset of sensory knobs from C57Bl6/NCrl mice that express Fpr3wt were activated, while knobs from 129X1/Sv mice that carry Fpr3Δ424-435 showed no responses. These results are consistent with a lack of receptor function in animals expressing Fpr3Δ424-435 caused by lack of four amino acids. Thus I conclude that Fpr3Δ424-435 is a non-functional Fpr3 variant, whereas Fpr3wt is a fully functional receptor variant. I further conclude that mice carrying Fpr3Δ424-435 lack Fpr3 function in general and thus provide functional knockout animals for Fpr3. Due to the lack of Fpr3 function, these mice likely suffer from impaired functions in the organs that express the receptor – the VNO and the immune system. However, the precise role of Fpr3 is not yet unraveled what makes it difficult to assess the kind of effect caused by the deletion. Moreover, my results demonstrate the detection of a pathogen-associated ligand by the VNO through Fpr3 arguing for an involvement of the intact receptor in vomeronasal pathogen-detection (see chapter 1.1).
4.3.2 Truncation of Fpr3Δ424-435 Protein Causes a Lack of Receptor Function

Because of the relatively small change of four amino acids, I initially expected both Fpr3 variants, Fpr3\textsubscript{wt} and Fpr3\textsubscript{Δ424-435}, to be functional. However, although the open reading frame remained intact, Fpr3\textsubscript{Δ424-435} lacked complete receptor function. Surprisingly, mRNA expression for both receptor variants was clearly demonstrated (see chapter 3.3.1) but receptor protein was only detectable for Fpr3\textsubscript{wt} (Figure 30). Thorough examination of Fpr3\textsubscript{Δ424-435} expression was performed to investigate the lack of receptor protein (see chapter 3.3.3).

Fpr3\textsubscript{Δ424-435} was not detectable in immunocytochemistry experiments using the newly generated antibodies, ECL1 and ECL2, in both HEK and dissociated VNO cells (Figures 26, 30). This argues that the 12 nucleotide in-frame deletion in Fpr3\textsubscript{Δ424-435} causes no or extremely diminished protein expression. An alternative explanation would be an altered protein structure that masks all antibody binding sites.

I generated a fusion-protein with an N-terminal Rho-tag and a C-terminal HSV-tag to evaluate if receptor protein was expressed at least partially (see chapter 2.3.11). Experiments with the resulting Rho-Fpr3\textsubscript{Δ424-435}-HSV construct revealed a clear but diminished staining for the Rho-tag antibody that bound N-terminally (Figure 31). Together with a lack of staining using the C-terminally binding HSV-tag antibody, the results indicate a truncated expression of Fpr3\textsubscript{Δ424-435} protein. The expressed protein included an N-terminus and discontinued somewhere before the C-terminus. I did not determine the exact extent to which the receptor was truncated but the lack of cellular staining with ECL1, which recognizes an epitope localized in front of the in-frame deletion (Figure 13), argues for a very short protein or severe misfolding of the resulting protein. The strongly reduced staining using the Rho-tag antibody argues for inefficient receptor synthesis or transport. Misfolded, damaged, and truncated proteins are prone to degradation via the ubiquitin-proteasome system (Goldberg, 2003; Bhattacharyya \textit{et al.}, 2014). This quality control system rapidly eliminates those proteins, as demonstrated early by the degradation of abnormally folded globin (Goldberg and Dice, 1974; Etlinger and Goldberg, 1976; Klemes \textit{et al.}, 1981; Sherman and Goldberg, 2001). Thus, reduced staining with the Rho-tag antibody is likely due to degradation of the truncated or misfolded receptor protein. However, the exact reason for the truncation and diminished protein amount has to be addressed in future experiments.

4.3.3 Distribution of the Fpr3 Variants Amongst Laboratory Mice

Genotyping combined with database analyses of the Fpr3 nucleotide sequence in different mouse strains revealed an astonishing number of mouse lines carrying the non-functional
DISCUSSION

The Fpr3Δ24-435 variant. Thus far, I identified 19 different strains encoding the Fpr3Δ24-435 variant. The functional Fpr3wt variant was present in 13 examined mouse strains. Surprisingly, the heredity transmission patterns of both receptor variants were inconsistent (Figure 40). The patterns show that Fpr3wt and Fpr3Δ24-435 were present in numerous laboratory mouse lines that originated either in Europe, North America, or Asia. Data from genetic analyses (Figures 27, 40) implied multiple places of origin for the Fpr3Δ24-435 variant. This finding argues for an independent emergence of Fpr3Δ24-435 in several founder mice.

Figure 40. Distribution of the Fpr3 variants in the laboratory mouse genealogy using the example of Castle’s mice. Distribution of strains expressing Fpr3wt (green) and Fpr3Δ24-435 (red) is inconsistent with any heredity transmission pattern. Strains of the same origin cluster, such as 129-related strains, seem to express the same variant. This argues for individual emergence of the Fpr3Δ24-435 variant and subsequent heredity. Strains not examined for their expressed variant are written in black letters. Figure after Beck et al., 2000.
One possible explanation for the inconsistent appearance of Fpr3Δ424-435 is occasional breeding with mice carrying this receptor variant. Gene refreshing by crosses with maximum genetic diversity to the foundation stocks is often performed between laboratory and wild living mice of common genetic background to prevent genetic drift in mouse breeding (Beck et al., 2000; Lambert, 2009). However, more laboratory strains expressing the non-functional Fpr3Δ424-435 variant rather than the functional Fpr3wt variant were identified (Figure 27). Although the distribution of Fpr3Δ424-435 in the laboratory mouse genealogy is wide and scattered (Figure 40), cluster-like heredity was partly observed. For instance, all examined 129-related mice expressed Fpr3Δ424-435. This argues for individual emergence of the Fpr3Δ424-435 variant and subsequent heredity. In this scenario the non-functional variant may have provided evolutionary benefits under breeding conditions. The loss of Fpr3 receptor function could have led to a positive selection of mice encoding Fpr3Δ424-435 during the breeding process by a phenotype that was favorable under laboratory conditions.

Mice can distinguish between healthy and infected individuals and avoid company of infected conspecifics dependent on a functioning VNO (Boillat et al., 2015). Laboratory mice may even stop breeding when in an unhealthy state (Lambert, 2009). My data from expression and functional experiments (see chapters 3.2 and 3.4) argue for the involvement of Fpr3 function in vomeronasal pathogen-detection. Thus, loss of Fpr3 function could have resulted in greater acceptance of non-suitable mating partners, food, bedding, and other breeding performance factors (see chapter 1.1). In line with this, several mouse strains, such as NFR/N mice, have become resistant to different types of stress, making them “high breeders” in most types of environmental conditions (Liljander et al., 2006). For these strains it is not examined which variant of Fpr3 they encode. However, mouse strains expressing the functional Fpr3wt, such as C57Bl1-related mice, normally produce lower numbers of litters and are often denoted as “moderate breeders” (Liljander et al., 2006). Mice experiencing less stress, e. g. through the lack of contact with sick conspecifics, produce more offspring. Thus, mice with an inhibited detection ability of stress factors through the lack of Fpr3 function could feature a better breeding phenotype in the laboratory breeding process. This argues for Fpr3 being a gene critical for reproduction in mice alongside already identified genes, such as Fecq1, Fecq2, and Ori (Kirkpatrick et al., 1998; Spearow and Barkley, 1999; Peripato et al., 2002; Peripato et al., 2004; Rocha et al., 2004; Everett et al., 2004).
4.3.4 Fpr3 Variants Originated in Wild Living Mice

One possible reason for the high frequency of the non-functional Fpr3Δ424-435 variant in laboratory mice was a frequent occurrence of Fpr3Δ424-435 in many wild-type mice. Mice of the species Mus musculus are believed to have originated in the north of the Indian subcontinent. From there they spread throughout the world probably 0.5 million years ago (Yonekawa et al., 1981; Boursot et al., 1993). Different colonization paths led to different local subspecies, such as Mus musculus castaneus in Southeast Asia, M. m. domesticus in Western Europe and the Mediterranean basin, and M. m. musculus in Central Europe and North China (Boursot et al., 1993). The genomes of most laboratory mouse strains comprise a mixed genetic background derived from these three subspecies (Beck et al., 2000).

Hence, I examined several wild-derived mouse strains that represent one of the individual subspecies M. m. castaneus, M. m. domesticus, and M. m. musculus, respectively. Nearly all examined wild-derived mouse strains carried the Fpr3wt gene (Figure 27). However, Fpr3Δ424-435 was present in three substrains of M. m. domesticus – LEWES/EiJ, WSB/EiJ, ZALENDE/EiJ – whereas two other substrains of M. m. domesticus – GER/DT, FRA/DT – carried the Fpr3wt variant. This result provides first evidence that strains from the subspecies M. m. domesticus are the original source of Fpr3Δ424-435 in laboratory mouse strains.

The genomes of many laboratory strains derived of only a few ancestral wild living strains with limited haplotype diversity (Tucker et al., 1992; Beck et al., 2000; Yang et al., 2011). In line with this, their genomes comprise mosaic patterns of different mus musculus subspecies (Bonhomme et al., 1987). Thus it is still possible that the Fpr3Δ424-435 gene emerged in different subspecies. A haplotype analysis of the Fpr3 gene in mouse strains carrying one of the two variants with the Mouse Phylogeny Viewer were performed to clarify the origin of Fpr3Δ424-435 (see chapter 2.8.2.5; Table 13; Appendix). This analysis gives information about from which founder mouse a defined gene locus in different laboratory mouse strains emerged (Yang et al., 2011). Most strains of either variant received their Fpr3 gene from M. m. domesticus. Only three other tested strains carrying Fpr3wt – C57BR/cdJ, C57L/J, C58/J – showed a M. m. musculus heredity. Intriguingly, all tested strains in which the non-functional Fpr3Δ424-435 variant was found, obtained their Fpr3 gene from a M. m. domesticus ancestor. This finding shows that the Fpr3Δ424-435 variant undoubtedly originated from the subspecies M. m. domesticus.

Different strains representing M. m. domesticus from different geographic regions were investigated for their encoded Fpr3 variant to determine which M. m. domesticus strain introduced Fpr3Δ424-435 into laboratory mice. Distribution of Fpr3Δ424-435 in wild-type
M. m. domesticus was highly unusual because it could not be retraced to a clear geographic origin. One strain from Switzerland and two others from North America carried Fpr3Δ424-435 whereas two other lines from Germany and France carried Fpr3wt (Figure 27). I expected that the non-functional Fpr3Δ424-435 was a rare incidence. However, the occurrence of Fpr3Δ424-435 in at least three wild living strains from geographically separated regions argues for multiple independent origins of Fpr3Δ424-435 under natural conditions.

Surprisingly, database analyses revealed that a wild-derived strain representing M. m. molossinus, which is believed to have solely originated from M. m. castaneus and M. m. musculus in Japan (Boursot et al., 1993; Beck et al., 2000; Yang et al., 2011) also expresses Fpr3Δ424-435 despite a geographic barrier that should prevent a cross-breeding with M. m. domesticus mice from North America or Europe.

4.4 Outlook

The functional orthology of murine Fpr3 to human FPR3 and upregulation of the receptor protein after stimulation with lipopolysaccharide in immune cells suggest a role for murine Fpr3 in the immune system and thus in pathogen detection. Furthermore, as bacterial signal peptides were detected by Fpr3-expressing vomeronasal sensory neurons, the receptor may be involved in pathogen detection by the vomeronasal organ. These findings pave the way for further investigations into the biological role of murine Fpr3.

The discovery of a multitude of natural Fpr3 knockout mouse strains provides a valuable tool to study murine Fpr3 function in the context of various genetic backgrounds. Thereby, comparative analyses of functional Fpr3wt and non-functional Fpr3Δ424-435 in native tissues will provide crucial information about the biological role of murine Fpr3.

To gain a deeper understanding of the biological role of Fpr3 in the immune system, calcium imaging experiments and assays including chemotaxis or release of reactive oxygen species could be performed with neutrophil granulocytes. However, no exclusive ligand for murine Fpr3 has been identified. Thus, identifying an exclusive Fpr3 ligand is an important step to shed light on the biological role of the receptor in the immune system. Moreover, neutrophil granulocytes express functionally promiscuous Fpr1 and Fpr2, which detect all known Fpr3 ligands with higher sensitivity than Fpr3. Thus, and because of the lack of reliable blockers for murine Fpr1 and Fpr2, definite answers about Fpr3 function in neutrophil granulocytes await the availability of such tools.
Fpr3 was not coexpressed with any other Fpr or other type of vomeronasal receptor in the vomeronasal organ (Liberles et al., 2009; Rivière et al., 2009). Thus, a detailed examination on the role of murine Fpr3 in the vomeronasal organ is possible. The main issue when investigating Fpr3 function in the vomeronasal organ is the small proportion of cells (0.3%) that express the receptor. This study introduced tools to unambiguously identify Fpr3-expressing cells. In cooperation with Dr. Andreas Schmid, I determined that mouse strains carrying Fpr3Δ424-435 had a functional Fpr3 knockout phenotype in the vomeronasal organ. Thus, Fpr3 function could be investigated by comparatively examining vomeronasal cells in strains expressing either Fpr3wt or Fpr3Δ424-435 with calcium imaging. To verify the functional data, Fpr3 expression in responding cells could be demonstrated by immunocytochemistry post-hoc using the newly generated ECL1 and ECL2 antibodies. In this context, the most interesting question is whether Fpr3 is actually involved in pathogen detection by the olfactory system. To this end, calcium imaging of vomeronasal cells and pathogen-related compounds in combination with a behavioral aversion assay (Boillat et al., 2015) could be performed. Thereafter, olfactory-based compounds would be of interest to evaluate adaptations of the receptor to the olfactory system.

A comparison of the behavior of mice carrying either Fpr3wt or Fpr3Δ424-435 would provide insight into the involvement of Fpr3 in general social behaviors mediated by the vomeronasal organ, such as aggressive or sexual behavior. However, based on the different genetic backgrounds of the mice, the results must be checked with Fpr3 knockout mice from the same genetic background.

This thesis reports the functional orthology of murine Fpr3 to human FPR3. Further studies should examine genes orthologous with murine Fpr3 in other species. Interesting questions include which functions of orthologous Fpr3 genes are conserved and if specific roles are associated with an olfactory phenotype. Fpr3 genes have been predicted in numerous apes and monkeys and the rat (NCBI data), but definite confirmation of receptor proteins remains to be shown. Thus, the first step to further evaluate Fpr3 orthology between species is to identify Fpr3 genes in more species. I speculate that Fpr3 genes will be identified in various rodents in the future. It would be interesting to compare Fpr3 function in animals with an intact vomeronasal organ to those without. To this end, functional measurements in vitro and in native tissues could be used as presented in this study.
5 LITERATURE


7. Bao L, Gerard NP, Eddy RL, Jr., Shows TB, Gerard C (1992) Mapping of genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. Genomics 13: 437-440


79. Gripentrog JM, Miettinen HM (2008) Formyl peptide receptor-mediated ERK1/2 activation occurs through G(i) and is not dependent on beta-arrestin1/2. Cell Signal 20: 424-431


160. Önheim K, Bylund J, Boulay F, Dahlgren C, Forsman H (2008) Tumour necrosis factor (TNF)-alpha primes murine neutrophils when triggered via formyl peptide receptor-related sequence 2, the murine orthologue of human formyl peptide receptor-like 1, through a process involving the type I TNF receptor and subcellular granule mobilization. Immunology 125: 591-600


175. Raisman G (1972) An experimental study of the projection of the amygdala to the accessory olfactory bulb and its relationship to the concept of a dual olfactory system. Exp Brain Res 14: 395-408


<table>
<thead>
<tr>
<th>Peptide#</th>
<th>Amino Acid#</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 to 15</td>
<td>W-E-T-T-E-D-V-D-V-D-V-M-G-D-V-V-Y</td>
</tr>
<tr>
<td>2</td>
<td>6 to 20</td>
<td>S-I-P-L-N-Q-S-D-V-V-I-Y-D-S-T</td>
</tr>
</tbody>
</table>
| 4        | 16 to 30    | I-Y-D-D-T-E-S-R-V-L-W-I-L-
| 5        | 21 to 35    | I-S-R-V-L-M-I-L-S-M-V-V-V-S-I |
| 6        | 26 to 40    | W-I-L-S-M-V-V-V-V-S-I-T-F-P-L-G |
| 7        | 31 to 45    | V-V-V-S-I-Y-F-P-L-G-V-V-L-G-H |
| 8        | 36 to 50    | T-F-P-L-G-V-V-L-G-V-V-I-Y-D-V-V |
| 10       | 46 to 60    | L-V-I-N-V-A-G-P-F-M-P-H-T-V-V-T |
| 11       | 51 to 65    | A-G-P-F-M-P-H-T-V-V-I-Y-D-S-T-L |
| 12       | 56 to 70    | P-H-V-T-V-T-I-V-W-V-L-H-L-A |
| 13       | 61 to 75    | T-V-N-Y-L-N-H-L-A-D-P-S-F-T-F-T |
| 15       | 71 to 85    | D-F-P-F-T-A-L-T-L-P-L-L-V-E-R-H |
| 18       | 86 to 100   | A-M-E-K-E-W-P-F-G-M-L-C-X-L |
| 19       | 91 to 105   | W-P-F-G-K-K-L-C-E-L-V-I-Y-D-V-V |
| 20       | 96 to 110   | F-L-L-I-L-V-L-I-L-V-V-D-V-V-V-F-L |
| 22       | 106 to 120  | D-V-V-L-F-G-V-V-P-L-I-A-V-I-A |
| 24       | 116 to 130  | I-A-V-V-L-A-D-R-C-1-C-V-L-F-P |
| 25       | 121 to 135  | L-D-R-C-C-I-C-V-L-H-P-V-V-A-Q-H |
| 26       | 126 to 140  | C-V-L-R-P-V-P-M-A-Q-N-H-R-V-T-V-S |
| 28       | 136 to 150  | R-B-R-V-T-V-L-A-R-V-V-V-V-G-M |
| 30       | 146 to 160  | V-V-V-S-W-I-P-A-L-I-L-V-I-L-P-L |
| 31       | 151 to 165  | I-P-A-L-I-L-I-T-V-L-P-L-P-F-T-I |
| 32       | 156 to 170  | L-T-L-L-L-P-L-L-P-L-T-V-V-V-V-R-A |
| 33       | 161 to 175  | F-L-F-L-T-T-V-R-T-A-G-R-G-V-V-H |
| 34       | 166 to 180  | T-V-V-D-A-R-V-G-V-V-H-C-R-L-S-F-V-S |
| 35       | 171 to 185  | B-G-D-V-D-V-L-L-V-R-V-D-V-V-V-V |
| 36       | 176 to 190  | C-B-R-L-V-V-V-V-G-D-V-V-E-V-E-X-L |
| 37       | 181 to 195  | V-S-V-G-S-V-S-V-E-V-E-R-L-H-N-A-T-A |
| 38       | 186 to 200  | S-V-V-E-R-L-N-T-A-I-T-P-V-T-T-T |
| 40       | 196 to 210  | T-F-P-V-T-T-V-T-G-I-I-R-F-I-V-V-F-F |
| 41       | 201 to 215  | B-G-I-I-I-R-F-I-V-V-S-F-S-L-M-P-H-S |
| 42       | 206 to 220  | F-I-V-S-P-S-I-L-P-M-S-V-V-A-I-C |
| 43       | 211 to 225  | S-L-P-H-R-F-V-A-I-C-D-G-L-L-T |
| 47       | 231 to 245  | B-A-F-V-S-V-S-D-V-F-V-V-L-G |
| 48       | 236 to 250  | S-S-P-R-F-V-V-V-V-G-V-V-A-A-F-F |
| 49       | 241 to 255  | B-V-L-T-P-V-V-V-A-A-F-F-V-C-I-C-H-F |
| 50       | 246 to 260  | V-V-A-S-P-P-F-I-C-W-F-P-P-Q-L-V-V |
| 51       | 251 to 265  | F-I-C-H-P-P-P-P-Q-L-V-L-A-L-L-L-T |
| 52       | 256 to 270  | P-F-P-P-L-V-L-L-L-L-L-V-V-V-V-L-E-C |
| 53       | 261 to 275  | A-L-L-G-V-V-V-V-M-E-F-K-M-D-P-F-G-S |
| 54       | 266 to 280  | V-H-L-C-K-E-H-Q-S-P-S-G-S-Y-K-V-I-I |
| 57       | 281 to 295  | G-R-L-V-N-P-T-S-S-L-A-P-P-S-H-S |
| 58       | 286 to 300  | P-T-S-S-L-A-P-P-H-S-C-S-L-N-P-H-S |
| 59       | 291 to 305  | A-P-P-D-P-S-C-I-L-P-I-L-V-V-P-M |
| 60       | 296 to 310  | C-L-H-P-I-I-T-V-Y-V-P-M-Q-D-D-G-O |
| 61       | 301 to 315  | L-Y-V-P-M-G-Q-Q-D-F-Q-E-R-L-I-H |
| 63       | 311 to 325  | E-R-L-I-H-L-I-L-S-R-L-D-G-D-A-Q-L-A |
| 64       | 316 to 330  | S-L-S-S-L-Q-R-A-L-L-S-E-D-S-G |
| 67       | 331 to 345  | H-I-D-D-P-P-T-D-V-L-A-D-P-L-F-R-D |
## Table 11. Mouse genes containing the AMKEK motif

<table>
<thead>
<tr>
<th>Gene#</th>
<th>Gene Name</th>
<th>Gene Synonyms</th>
<th>Locus</th>
<th>Amino Acids</th>
<th>UniProtKB Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cede83</td>
<td>-/-</td>
<td>CCD83_MOUSE</td>
<td>305 aa</td>
<td>Q9DI4V3.1</td>
</tr>
<tr>
<td>2</td>
<td>Fpr2</td>
<td>Fpr-rs2</td>
<td>FPR2_MOUSE</td>
<td>351 aa</td>
<td>Q85536.1</td>
</tr>
<tr>
<td>3</td>
<td>Fpr3</td>
<td>Fpr-rs1; Lxa4r</td>
<td>FPRS1_MOUSE</td>
<td>351 aa</td>
<td>Q08790.2</td>
</tr>
<tr>
<td>4</td>
<td>Map9</td>
<td>Asap; Mtap9</td>
<td>MAP9_MOUSE</td>
<td>646 aa</td>
<td>Q3TRRR0.2</td>
</tr>
<tr>
<td>5</td>
<td>Skil</td>
<td>Skir; Sno</td>
<td>SKIL_MOUSE</td>
<td>675 aa</td>
<td>Q6O665.2</td>
</tr>
<tr>
<td>6</td>
<td>Tsga10</td>
<td>-/-</td>
<td>TSG10_MOUSE</td>
<td>697 aa</td>
<td>Q6NY15.1</td>
</tr>
<tr>
<td>7</td>
<td>Lppr3</td>
<td>Kiaa0476, Prg2</td>
<td>LPPR3_MOUSE</td>
<td>716 aa</td>
<td>Q7TCP8.1</td>
</tr>
<tr>
<td>8</td>
<td>Elmo2</td>
<td>Kiaa1834</td>
<td>ELMO2_MOUSE</td>
<td>732 aa</td>
<td>Q8BHL5.1</td>
</tr>
<tr>
<td>9</td>
<td>Nup98</td>
<td>-/-</td>
<td>NUP98_MOUSE</td>
<td>1816 aa</td>
<td>Q69FD9.2</td>
</tr>
<tr>
<td>10</td>
<td>Unc13c</td>
<td>-/-</td>
<td>UN13C_MOUSE</td>
<td>2210 aa</td>
<td>Q8KOT7.3</td>
</tr>
<tr>
<td>11</td>
<td>Tpr</td>
<td>-/-</td>
<td>TPR_MOUSE</td>
<td>2431 aa</td>
<td>F6ZDS4.1</td>
</tr>
<tr>
<td>12</td>
<td>Pcnt</td>
<td>Pcnt2</td>
<td>PCNT_MOUSE</td>
<td>2898 aa</td>
<td>P48725.2</td>
</tr>
<tr>
<td>13</td>
<td>Akap9</td>
<td>Kiaa0803</td>
<td>AKAP9_MOUSE</td>
<td>3797 aa</td>
<td>Q7P0F1.2</td>
</tr>
<tr>
<td>14</td>
<td>Dnha8</td>
<td>Dnha8</td>
<td>DYH8_MOUSE</td>
<td>4731 aa</td>
<td>Q91XQ0.2</td>
</tr>
<tr>
<td>15</td>
<td>Fsp2</td>
<td>-/-</td>
<td>FSIP2_MOUSE</td>
<td>6995 aa</td>
<td>A2ARZ3.3</td>
</tr>
<tr>
<td>16</td>
<td>Dist</td>
<td>Bpag1; Macf2</td>
<td>DYST_MOUSE</td>
<td>7393 aa</td>
<td>Q912U6.2</td>
</tr>
<tr>
<td>17</td>
<td>Synel</td>
<td>-/-</td>
<td>SYNE1_MOUSE</td>
<td>8799 aa</td>
<td>Q6ZWR6.2</td>
</tr>
</tbody>
</table>

## Table 12. Mouse genes containing the LNTA motif

<table>
<thead>
<tr>
<th>Gene#</th>
<th>Gene Name</th>
<th>Gene Synonyms</th>
<th>Locus</th>
<th>Amino Acids</th>
<th>UniProtKB Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Atoh7</td>
<td>Ath5</td>
<td>ATOH7_MOUSE</td>
<td>149 aa</td>
<td>Q92Z3E.1</td>
</tr>
<tr>
<td>2</td>
<td>Ormdl1</td>
<td>-/-</td>
<td>ORML1_MOUSE</td>
<td>153 aa</td>
<td>Q2118.1</td>
</tr>
<tr>
<td>3</td>
<td>Ucn3</td>
<td>-/-</td>
<td>UCN3_MOUSE</td>
<td>164 aa</td>
<td>Q924A4.1</td>
</tr>
<tr>
<td>4</td>
<td>Ptn</td>
<td>-/-</td>
<td>PTN_MOUSE</td>
<td>168 aa</td>
<td>P63089.1</td>
</tr>
<tr>
<td>5</td>
<td>Fam159a</td>
<td>-/-</td>
<td>F159A_MOUSE</td>
<td>189 aa</td>
<td>A2A9G7.1</td>
</tr>
<tr>
<td>6</td>
<td>Isoc2a</td>
<td>Isoc2</td>
<td>ISC2A_MOUSE</td>
<td>206 aa</td>
<td>P85094.1</td>
</tr>
<tr>
<td>7</td>
<td>Ropn11</td>
<td>Asp</td>
<td>ROP11_MOUSE</td>
<td>230 aa</td>
<td>Q9EQ00.1</td>
</tr>
<tr>
<td>8</td>
<td>Tmem65</td>
<td>-/-</td>
<td>TMM65_MOUSE</td>
<td>234 aa</td>
<td>Q4VAE3.1</td>
</tr>
<tr>
<td>9</td>
<td>Bpifa2</td>
<td>Psp</td>
<td>BPIA2_MOUSE</td>
<td>235 aa</td>
<td>P07743.1</td>
</tr>
<tr>
<td>10</td>
<td>Pex11g</td>
<td>Pex11c</td>
<td>PX11C_MOUSE</td>
<td>241 aa</td>
<td>Q6PM5.2</td>
</tr>
<tr>
<td>11</td>
<td>Drgx</td>
<td>Drg11; Prx11</td>
<td>DRGX_MOUSE</td>
<td>263 aa</td>
<td>Q8BYH0.2</td>
</tr>
<tr>
<td>12</td>
<td>Mad2l1hp</td>
<td>Mad2l1p</td>
<td>MD2BP_MOUSE</td>
<td>276 aa</td>
<td>Q9DCX1.2</td>
</tr>
<tr>
<td>13</td>
<td>Elov17</td>
<td>-/-</td>
<td>ELOV7_MOUSE</td>
<td>281 aa</td>
<td>Q9D2Y9.1</td>
</tr>
<tr>
<td>14</td>
<td>Mbd3</td>
<td>-/-</td>
<td>MBD3_MOUSE</td>
<td>285 aa</td>
<td>Q92ZD6.1</td>
</tr>
<tr>
<td>15</td>
<td>Raly</td>
<td>-/-</td>
<td>RALY_MOUSE</td>
<td>293 aa</td>
<td>Q8BF8.1</td>
</tr>
<tr>
<td>16</td>
<td>Rally</td>
<td>Merc</td>
<td>RALY_MOUSE</td>
<td>312 aa</td>
<td>Q6Q12.3</td>
</tr>
<tr>
<td>17</td>
<td>Cede160</td>
<td>-/-</td>
<td>CCI160_MOUSE</td>
<td>323 aa</td>
<td>Q3UYG1.1</td>
</tr>
<tr>
<td>18</td>
<td>Cdk6</td>
<td>Cdkn6; Crk2</td>
<td>CDK6_MOUSE</td>
<td>326 aa</td>
<td>Q64261.2</td>
</tr>
<tr>
<td>19</td>
<td>Ufl1</td>
<td>-/-</td>
<td>UFI1_MOUSE</td>
<td>339 aa</td>
<td>Q3H1H4.2</td>
</tr>
<tr>
<td>20</td>
<td>Gpr139</td>
<td>Gm495; Gprg1; Pgr3</td>
<td>GP139_MOUSE</td>
<td>345 aa</td>
<td>Q8U0CF.2</td>
</tr>
<tr>
<td>21</td>
<td>Fkbp1</td>
<td>Ng7</td>
<td>FKBP1_MOUSE</td>
<td>347 aa</td>
<td>Q35450.1</td>
</tr>
<tr>
<td>22</td>
<td>Fpr2</td>
<td>Fpr-rs2</td>
<td>FPR2_MOUSE</td>
<td>351 aa</td>
<td>Q85536.1</td>
</tr>
<tr>
<td>23</td>
<td>Fpr3</td>
<td>Fpr-rs1; Lxa4r</td>
<td>FPRS1_MOUSE</td>
<td>351 aa</td>
<td>Q08790.2</td>
</tr>
<tr>
<td>24</td>
<td>Tefm</td>
<td>-/-</td>
<td>TEFM_MOUSE</td>
<td>364 aa</td>
<td>Q5SS3K.1</td>
</tr>
<tr>
<td>25</td>
<td>Tm6sf1</td>
<td>-/-</td>
<td>TM6S1_MOUSE</td>
<td>370 aa</td>
<td>P58749.2</td>
</tr>
<tr>
<td>26</td>
<td>Sox18</td>
<td>Sox-18</td>
<td>SOX18_MOUSE</td>
<td>377 aa</td>
<td>P43680.3</td>
</tr>
<tr>
<td>27</td>
<td>Stom1</td>
<td>-/-</td>
<td>STML1_MOUSE</td>
<td>399 aa</td>
<td>Q8C166.1</td>
</tr>
<tr>
<td>28</td>
<td>Ager</td>
<td>Rage</td>
<td>RAGE_MOUSE</td>
<td>403 aa</td>
<td>Q62151.1</td>
</tr>
<tr>
<td>29</td>
<td>Slc22a18</td>
<td>Imp1; Itm; Oel2; Tssc5</td>
<td>S22A1_MOUSE</td>
<td>406 aa</td>
<td>Q78K3K.2</td>
</tr>
<tr>
<td>30</td>
<td>Tnmlhe</td>
<td>Tnml</td>
<td>TNLH_MOUSE</td>
<td>421 aa</td>
<td>Q912E9.2</td>
</tr>
<tr>
<td>31</td>
<td>Pmm20d2</td>
<td>Acyl12; Gm424</td>
<td>P20D2_MOUSE</td>
<td>431 aa</td>
<td>A3KG59.1</td>
</tr>
<tr>
<td>32</td>
<td>Gcddh</td>
<td>-/-</td>
<td>GCDDH_MOUSE</td>
<td>438 aa</td>
<td>Q60759.2</td>
</tr>
<tr>
<td>33</td>
<td>Midn</td>
<td>-/-</td>
<td>MIDN_MOUSE</td>
<td>465 aa</td>
<td>Q3TP7.1</td>
</tr>
<tr>
<td>34</td>
<td>Phip2</td>
<td>Ptp2</td>
<td>PTP2_MOUSE</td>
<td>468 aa</td>
<td>P17892.1</td>
</tr>
<tr>
<td>35</td>
<td>Gtr2a11</td>
<td>Alf; Gt2a1lf</td>
<td>TF2AY_MOUSE</td>
<td>468 aa</td>
<td>Q8R4AR.2</td>
</tr>
<tr>
<td>36</td>
<td>Amigo1</td>
<td>Ali2; Amigo; Kiaa163</td>
<td>AMIGO1_MOUSE</td>
<td>492 aa</td>
<td>Q80ZD6.1</td>
</tr>
<tr>
<td>37</td>
<td>Phf10</td>
<td>Baf45a</td>
<td>PHF10_MOUSE</td>
<td>497 aa</td>
<td>Q9D8M7.4</td>
</tr>
</tbody>
</table>
Appendix

| 38 | Celf2 | Cagbp2; Napor | CELF2_MOUSE | 508 aa | Q9Z0H4.1 |
| 39 | Apex2 | Ape2 | APEX2_MOUSE | 516 aa | Q68G58.1 |
| 40 | Snx1 | -/- | SNX1_MOUSE | 522 aa | Q9WV80.1 |
| 41 | Ppp3cb | Calnb | PPBB_MOUSE | 525 aa | P48453.2 |
| 42 | Fgfr1 | Fgfr5 | FGR1_MOUSE | 529 aa | Q91V87.1 |
| 43 | Cdec176 | Bbof1 | BBOF1_MOUSE | 533 aa | Q3V079.2 |
| 44 | Nrbp1 | Madm; Nrbp | NRBP_MOUSE | 535 aa | Q99345.1 |
| 45 | S1c2a10 | Glut10 | GTR10_MOUSE | 536 aa | Q8VHD6.1 |
| 46 | Stm | Stm1 | STAM1_MOUSE | 548 aa | P70297.3 |
| 47 | Tbx4 | -/- | TBX4_MOUSE | 552 aa | P70325.3 |
| 48 | Mstoi | -/- | MTO1_MOUSE | 556 aa | Q2YD2W.1 |
| 49 | Fvcr1 | Mfsd7b | FLVC1_MOUSE | 560 aa | B2RXV4.1 |
| 50 | Cdc6 | -/- | CDC6_MOUSE | 562 aa | O89033.2 |
| 51 | Deaf1 | -/- | DEAF1_MOUSE | 566 aa | Q9ZI7T.1 |
| 52 | Znu704 | Gigt1; Zifp704 | ZN704_MOUSE | 566 aa | Q9ERQ3.1 |
| 53 | Cerc6 | -/- | CERC6_MOUSE | 572 aa | Q99MXT.1 |
| 54 | Taf5i | Paf65b | TAF5L_MOUSE | 589 aa | Q91WQ5.1 |
| 55 | Rangap1 | Fug1 | RAGP1_MOUSE | 589 aa | P46061.1 |
| 56 | Khlh10 | -/- | KHLH10_MOUSE | 608 aa | Q9DSV2.1 |
| 57 | Hap1 | -/- | HAP1_MOUSE | 628 aa | O35668.1 |
| 58 | Vps9d1 | -/- | VPS9D1_MOUSE | 649 aa | Q8C190.1 |
| 59 | Znu746 | Zifp746 | ZN746_MOUSE | 652 aa | Q3U133.3 |
| 60 | Mepce | Bcdin3; Bipl1; D5Swu46e | MEPECE_MOUSE | 666 aa | Q8K39A.2 |
| 61 | Eri2 | Exod1; Kaa1504 | ER2_MOUSE | 688 aa | Q5BK4S.1 |
| 62 | Polh | Rad30a; Xpv | POLH_MOUSE | 694 aa | Q9JN0.1 |
| 63 | Ddx4 | Vasa | DDX4_MOUSE | 702 aa | Q61496.2 |
| 64 | Ninf | -/- | NEUL_MOUSE | 704 aa | Q9Y1YP.1 |
| 65 | Gga3 | Kiaa0154 | GGA3_MOUSE | 718 aa | Q8BM13.2 |
| 66 | Mtfl2 | -/- | IF2M_MOUSE | 727 aa | Q91YJS.2 |
| 67 | Prox1 | -/- | PROX1_MOUSE | 737 aa | P48437.2 |
| 68 | Cnct10 | -/- | CNCT10_MOUSE | 744 aa | Q8BH15.1 |
| 69 | Hectd2 | -/- | HECDT2_MOUSE | 774 aa | Q8C9U6.2 |
| 70 | Zwo1 | -/- | ZW10_MOUSE | 779 aa | O54692.3 |
| 71 | Sp4 | -/- | SP4_MOUSE | 782 aa | Q62445.2 |
| 72 | Vps35 | Mem3 | VPS35_MOUSE | 796 aa | Q9E0Q3.1 |
| 73 | Rapgef5 | Gfr; Kiaa0277; Mrgef | RPGF5_MOUSE | 814 aa | Q8COQ9.2 |
| 74 | Bicd1 | -/- | BICD1_MOUSE | 835 aa | Q8RRO7.2 |
| 75 | Kdm1a | Aof2; Kiaa0601; Lsd1 | KDM1A_MOUSE | 853 aa | Q62QQ8.2 |
| 76 | Ppp1r10 | Cat53; Pnuts | PPIA_MOUSE | 888 aa | Q8O0W0.1 |
| 77 | Actn1 | -/- | ACTN1_MOUSE | 892 aa | Q7TPR4.1 |
| 78 | Ctna3 | Ctna3 | CTNA3_MOUSE | 895 aa | Q65CL1.2 |
| 79 | Actn3 | -/- | ACTN3_MOUSE | 900 aa | Q88990.1 |
| 80 | unknown | -/- | CA112_MOUSE | 903 aa | Q3TQQ9.2 |
| 81 | Mcm2 | Bm28; Cdc11; Kiaa0030; Mcmd2 | MCM2_MOUSE | 904 aa | P97310.3 |
| 82 | Magee1 | -/- | MAGE1_MOUSE | 918 aa | Q8PCZ4.1 |
| 83 | Paxbp1 | Gfcb; Gfcb1 | PAXB1_MOUSE | 919 aa | P58501.3 |
| 84 | Tex11 | -/- | TEX11_MOUSE | 947 aa | Q14AT2.1 |
| 85 | Pwi12 | Mili | PIW1L2_MOUSE | 971 aa | Q8CDG1.2 |
| 86 | Nkb1 | -/- | NFKB1_MOUSE | 971 aa | P25799.2 |
| 87 | Imp5b | -/- | ISP2_MOUSE | 993 aa | Q8K337.1 |
| 88 | Demd1a | -/- | DEN1A_MOUSE | 1016 aa | Q8K382.2 |
| 89 | Paxip1 | Ptip | PAXI1_MOUSE | 1056 aa | Q6NZQ4.1 |
| 90 | Hip1r | -/- | HIP1R_MOUSE | 1068 aa | Q9JKYS.2 |
| 91 | Rimb2 | Kiaa0318; Rbp2 | RIMB2_MOUSE | 1072 aa | Q80U40.3 |
| 92 | Tmem152d | Molt | T132D_MOUSE | 1097 aa | Q76HP3.1 |
| 93 | Tmem152c | -/- | T132C_MOUSE | 1099 aa | Q8CEF9.3 |
| 94 | Pkp4 | Armmp | PKP4_MOUSE | 1190 aa | Q68F0H.1 |
| 95 | Kch7 | Erg3 | KCNH7_MOUSE | 1195 aa | Q9ER47.2 |
| 96 | Egfr | -/- | EGFR_MOUSE | 1210 aa | Q01279.1 |
| 97 | Tdrd12 | Ecath6; Repro23 | TDR12_MOUSE | 1215 aa | Q6CWU0.2 |
| 98 | Evc2 | Lbn | LBN_MOUSE | 1220 aa | Q8K1G2.1 |
| 99 | Gigy2 | Kiaa0642; Perq2; Tnec15 | PERQ2_MOUSE | 1291 aa | Q8Y7W8.2 |
| 100 | Disp2 | Kiaa1742 | DISP2_MOUSE | 1345 aa | Q8CIP5.1 |
Table 13. Haplotype analysis of the {Fpr3} gene in different mouse strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Fpr3 Variant</th>
<th>Subspecific Origin of Fpr3</th>
<th>Origin</th>
<th>Inbred Strain Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6NJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>C57Bl/6Nj</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>C3</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>A/J</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>BALB/cJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>C57Bl/10J</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>NZB/6JxJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>NZB/6xLyJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>NZW/LacJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>England</td>
<td>Castle's mice</td>
</tr>
<tr>
<td>KK/HJ&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Japan</td>
<td>Colonies from China and Japan</td>
</tr>
<tr>
<td>NOD/ShIJo</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Switzerland</td>
<td>Swiss mice</td>
</tr>
<tr>
<td>ST/J</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>unknown</td>
<td>Other inbred strains</td>
</tr>
<tr>
<td>C57Bl/6N1N16</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spain</td>
</tr>
<tr>
<td>C3H/HeJc</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C3H/HeJj</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C3H/HeJn</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C57Bl/6N1J</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C57Bl/10JxJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>NZB/6JxJxJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>NZW/LacJxJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>KK/HJ&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>NOD/ShIJo</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>ST/J</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>unknown</td>
<td>Other inbred strains</td>
</tr>
<tr>
<td>C57Bl/6N1N16</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C3H/HeJc</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C3H/HeJj</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C3H/HeJn</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>C57Bl/10JxJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>NZB/6JxJxJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>NZW/LacJxJ</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>KK/HJ&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>NOD/ShIJo</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>Spain</td>
<td>Spanish strains</td>
</tr>
<tr>
<td>ST/J</td>
<td>Fpr3&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>M. m. domesticus</td>
<td>unknown</td>
<td>Other inbred strains</td>
</tr>
</tbody>
</table>

Subspecific origins were examined on chromosome 17 in the nucleotide range from 17,970,000 to 17,972,000. n. e. = not examined.
Publications

Journal Articles


Stempel H, Zufall F, Bufe B. Evidence for an Orthologous Function of Mouse and Human Formyl Peptide Receptor 3. In preparation

Abstracts

Stempel H, Schumann T, Bufe B, Zufall F
RNA editing alters the function of vomeronasal formyl peptide receptors.
29.08.2013: 22nd Annual Meeting of the European Chemoreception Research Organization

Bufe B, Schumann T, Stempel H, Zufall F
Evolution of formyl peptide receptor function in mammals
05.11.2012: 17th Joint Meeting of the Signal Transduction Society

Schumann T, Stempel H, Chamero P, Leinders-Zufall T, Zufall F, Bufe B
Formyl peptide detection by vomeronasal sensory neurons is mediated by functionally distinct cell populations
07.09.2012: Sonderforschungsbereich 894 Symposium on Calcium Signaling

Bufe B, Schumann T, Stempel H, Zufall F
Agonist profiling of the mouse formyl peptide receptors reveals a stereoselective tuning of mFpr-rs1
27.06.2012: 16th International Symposium on Olfaction and Taste
Copyright Permission Policy of the
American Society for Biochemistry and Molecular Biology

These guidelines apply to the reuse of articles, figures, charts and photos in the *Journal of Biological Chemistry, Molecular & Cellular Proteomics* and the *Journal of Lipid Research*.

For authors reusing their own material:
Authors need NOT contact the journal to obtain rights to reuse their own material. They are automatically granted permission to do the following:

- Reuse the article in print collections of their own writing.
- Present a work orally in its entirety.
- Use an article in a thesis and/or dissertation.
- Reproduce an article for use in the author's courses. (If the author is employed by an academic institution, that institution also may reproduce the article for teaching purposes.)
- Reuse a figure, photo and/or table in future commercial and noncommercial works.
- Post a copy of the paper in PDF that you submitted via BenchPress.
- Link to the journal site containing the final edited PDFs created by the publisher.
- Authors who published their papers under the “Author's Choice” option may post the final edited PDFs created by the publisher to their own/departmental/university Web sites immediately upon publication. All other authors may do so 12 months after publication.

EXCEPTION: If authors select the Author’s Choice publishing option:

- The final version of the manuscript will be covered under the Creative Commons Attribution license (CC BY), the most accommodating of licenses offered.
- The final version of the manuscript will be released immediately on the publisher’s website and PubMed Central.

Please note that authors must include the following citation when using material that appeared in an ASBMB journal:

"This research was originally published in Journal Name. Author(s). Title. *Journal Name*. Year; Vol:pp-pp. © the American Society for Biochemistry and Molecular Biology."

Updated May 13, 2016

Citation:

Einverständniserklärung

Ich bin damit einverstanden, dass Herr Hendrik Stempel die von ihm angegebenen Daten zur inhaltlichen Darstellung seiner Dissertation verwenden darf. Die Daten wurden unentgeltlich erstellt und freiwillig zur Verwendung genehmigt.

(1) Bilder der Original Peptid Spot-Arrays der Antikörper M-20, ECL1 und ECL2.

Mit freundlicher Genehmigung: Martin Jung

(2) Messung von Calciumsignalen in sensorischen Köpfen in whole mount Präparationen des Vomeronasalorgans von C57Bl/6NCrl und 129X1/Sv Mäusen ausgelöst durch Salmonella-SP24.

Mit freundlicher Genehmigung: Andreas Schmid

Alle Angaben wurden nach bestem Wissen und Gewissen erstellt.
Acknowledgements

First of all, I want to thank Prof. Dr. Dr. Frank Zufall for giving me the opportunity to work on a challenging doctoral thesis that combined interesting questions and diverse methodology. Through working on this thesis I gained insight into many facets of science and had the opportunity to develop myself as a scientist as well as a person. Further, I would like to thank him for the support of my project with inspiring discussions, smooth access to expensive working material, and funding my project. I also thank him for enabling me to travel to a scientific meeting and associated with that the opportunity to present and discuss my work with leading international scientists. Finally, I thank him for reviewing this thesis as 1st reviewer.

I am very grateful to Dr. Bernd Bufe for his commendable supervision. His always friendly mentoring and advice helped me a lot during the laboratory work as well as the writing of publications and this thesis. Our encouraging and insightful discussions allowed me to continually develop and improve my skills in all requirements of science, be it strategic experimentation, time scheduling, scientific writing, or critical thinking. I appreciate his open-mindedness and his benevolent support for external advanced trainings. Finally, I thank him for thoroughly proofreading this thesis.

I thank PD. Dr. Martin Jung for the kind collaboration and, as part of this, for performing the peptide-spot array experiments for antibody characterization. Further, I thank him for supporting me with friendly and valuable scientific advice and for many inspiring conversations. Finally, I appreciate his willingness to review this thesis as 2nd reviewer.

I thank PD. Dr. Andreas Schmid for the permission to use in vivo calcium imaging data on whole mount preparations of the sensory knob side of VNOs of C57Bl/6NCr1 and 129X1/Sv mice with Salmonella-SP24 in this thesis. I also thank him for teaching me how to perform a whole mount preparation of the vomeronasal organ and for his support in my attempts to stain single dendritic knobs of Fpr3-expressing cells with my self-generated antibodies.
I am very grateful to Sabine Plant for her always friendly and benevolent technical support in many issues. Our very nice and humanly conversations often have shortened the time of experiments. Furthermore, I thank her for sharing her professional expertise in heterologous cell culture and her dedication in solving upcoming problems. Finally, I thank her for the thawing and cultivating cells, collecting of antibody supernatants, and purifying plasmids from time to time.

I thank Prof. Dr. Trese Leinders-Zufall for her friendly ear and her commitment for all of her students. Further, I thank her for providing me with C57Bl/6NCrl (MGI:2683688), BALB/cJ (MGI:2159737), and 129X1/Sv (MGI:2164536) mice. I also thank the staff of the animal facility, Andrea Degreif, Angelika Ströer, Sarah Boll, Monika Vorndran, Kerstin Becker, and Lisa Knieriemen for the animal caretaking and their friendly and professional support in all issues regarding mice.

I thank Prof. Dr. Frank Kirchhoff for providing me with FVB/NCrl (MGI:2165215) mice. In this context, I would like to thank Dr. Anja Scheller and Daniel Rhode for their uncomplicated and kind help on organizing mice from the animal facility of Prof. Dr. Frank Kirchhoff.

I thank Prof. Dr. Dieter Bruns for providing me with C57Bl/6NCrl (MGI:2683688) mice. I thank Marina Wirth und Judith Arend for their always kind and reliable help on organizing mice from the animal facility of Prof. Dr. Dieter Bruns.

I thank Prof. Dr. Diethard Tautz from the Max-Planck-Institut für Evolutionsbiologie in Plön, Germany for kindly providing me with ear stamps of wild-derived mouse strains. These included Mus musculus musculus (Kazakhstan, Czech Republic), M. m. domesticus (Germany, France), M. m. castaneus (Thailand), and M. m. spretus (Spain).

I would like to thank Dr. Reinhart Kluge from the Max Rubner Laboratorium at the German Institute of Human Nutrition, Potsdam Rehbrücke, Germany for kindly providing me with NZB/Ola (MGI:2160555) mice.
I would like to thank Dr. Anabel Pérez-Gómez for her scientific advice in many issues and for introducing me into the method of performing immunostainings on dissociated vomeronasal cells.

I thank Dr. Martina Pyrski for her scientific advice in complicated issues and many nice conversations.

I thank Dr. Pablo Chamero for his scientific advice in many issues.

I thank Marta Podgórska for helping me with the assessment of the purity of my human neutrophil preparations with FAC sorting.

I thank Petra Hammes for providing me with antibodies, technical advice in some issues and many nice conversations.

I thank Gabriele Mörschbächer for helping and advising me with all kinds of bureaucracy during the thesis.

I thank Holger Frisch for his always kind and competent help with IT-problems and for his very refreshing dry humor.

I also thank my fellow students Dr. Timo Schumann, Benjamin Stein, Florian Bolz, Eugenia Eckstein, Dennis Bakker, and Henrike Reder for many lovely conversations and support in all circumstances. I also want to thank Katherin Bleymehl, Michael Dieli, Dr. Christian Schauer, Yannick Teuchert, Thomas Blum, and Tong Tong for the always benevolent support among the students. I want to thank Benjamin Stein for introducing me to the method of vomeronasal cell dissociation.

Last but not least, I am grateful to my family and all my friends who supported and motivated me in situations of life over the years spent on this thesis.

Danke.