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Title

Amyloid beta and its naturally occurring N-terminal variants are potent activators of human and mouse formyl peptide receptor 1

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Formyl peptide receptors in amyloid beta sensing

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Abstract
Formyl peptide receptors (FPR) may contribute to inflammation in Alzheimer’s disease (AD) through interactions with neuropathological Amyloid beta (Aβ) peptides. Previous studies reported activation of FPR2 by Aβ1-42, but further investigation of other FPRs and Aβ variants is needed. This study provides a comprehensive overview of the interactions of mouse and human FPRs with different physiologically relevant Aβ-peptides using transiently transfected cells in combination with calcium imaging. We observed that, in addition to hFPR2, all other hFPRs also responded to Aβ1-42, Aβ1-40, and the naturally occurring variants Aβ11-40 and Aβ17-40. Notably, Aβ11-40 and Aβ17-40 are very potent activators of mouse and human FPR1, acting at nanomolar concentrations. Buffer composition and aggregation state are extremely crucial factors that critically affect the interaction of Aβ with different FPR subtypes. To investigate the physiological relevance of these findings, we examined the effects of Aβ11-40 and Aβ17-40 on the human glial cell line U87. Both peptides induced a strong calcium flux at concentrations that very similar to those obtained in experiments for hFPR1 in HEK cells. Further immunocytochemistry, qPCR, and pharmacological experiments verified that these responses were primarily mediated through hFPR1. Chemotaxis experiments revealed that Aβ11-40 but not Aβ17-40 evoked cell migration,
which argues for a functional selectivity of different Aβ peptides. Together, these findings provide the first evidence that not only hFPR2 but also hFPR1 and hFPR3 may contribute to neuroinflammation in AD through an interaction with different Aβ variants.

**Introduction**

Alzheimer’s disease (AD) is a complex neurodegenerative disorder with a heterogeneous pathobiology leading to progressive dementia with death as an inevitable outcome, generally within 5–12 years after symptom onset. While there is an urgent need for therapies that may prevent or slow the progression of AD, no unequivocal treatment is currently available. A better understanding of the molecular mechanisms underlying AD can help to identify new strategies to develop such treatments. The discovery that extracellular amyloid beta (Aβ) depositions and intracellular accumulation of hyperphosphorylated tau are common neuropathological hallmarks of all AD forms were significant advances that helped to clarify several key aspects of the underlying pathology. Although these discoveries are already more than three decades old, the precise impact of Aβ on neuroinflammation and neurodegeneration is still incompletely understood. Major challenges in Aβ research comprise the complex peptide processing involving multiple proteases, the challenging physiochemical properties of the resulting fragments that permit a formation of different oligomeric structures, and the pleiotropic physiological effects of Aβ-peptides on neurons, glia, and immune cells.

Aβ is produced by a sequential cleavage of an amyloid precursor protein (APP) by β- and γ-secretase. Cleavage can occur at several sites, which results in the predominant production of peptides with 38-, 40-, and 42-amino acid length. APP processing is somewhat imprecise and thus, depending on the specific (patho)physiological conditions, several additional longer and shorter Aβ variants in varying concentrations can occur. Many of them tend to form β sheet conformations that are prone to self-aggregate into different dimers, trimers, and tetramers, higher-order oligomers, protofibrils, and ultimately, typical 8 nm amyloid fibrils. There is clear evidence that the precise composition of these aggregates has a strong impact on their neurotoxicity and that different Aβ variants can trigger varying amounts of detrimental pro-inflammatory activities in astrocytes and microglia. Their biochemical properties can vary significantly depending on the microenvironment in which they are generated, their amino acid composition and their carboxyl terminus. This makes the precise assessment of the Aβ structure for the etiology of AD extremely difficult.

Microglia are a key factor for AD. In a healthy brain, they provide tropic support to neurons while simultaneously surveying the CNS for pathological stimuli. Upon activation, they undergo morphological changes and assume a reactive phenotype where they cease their supportive role. Instead, they obtain phagocytic and inflammatory functions, start to produce pro-inflammatory
cytokines and chemokines such as IL-1β, IL-6, IL-8 and TNFα and generate oxidative stress through the release of nitric oxide (NO) and reactive oxygen species (ROS)\textsuperscript{14,18}. Small oligomeric variants of Aβ are potent activators of microglia that lead to more severe neurotoxic outcomes than larger variants\textsuperscript{19,20}. It is therefore crucial to understand how different Aβ variants interact with microglia to produce these neurotoxic effects. Unfortunately, interactions between Aβ, and microglial cells are highly complex because Aβ acts through multiple pathways including TREM2 (Triggering receptor expressed on myeloid cells 2), TRPM2 (Transient receptor potential cation channel, subfamily M, member 2)\textsuperscript{21}, scavenger receptors such as CD36, MARCO (Macrophage receptor with collagenous structure) and RAGE (receptor for advanced glycation endproducts)\textsuperscript{22} and pattern recognition receptors (PRR) such as TLR (Toll-like receptors)\textsuperscript{23}, NLRs (NOD-like receptors)\textsuperscript{24} and FPRs (formyl peptide receptors)\textsuperscript{12}. The activation of PRRs by Aβ has become a recent focus in AD research because they have a special ability to potently trigger the reactive state\textsuperscript{14,25}. Among these PRRs, FPRs are one of the promising targets for AD research\textsuperscript{12}. They belong to a small gene family of G protein coupled receptors with three members in humans and seven in mice\textsuperscript{26}. FPRs are primarily expressed in the innate and adaptive immune system, where they contribute to the detection and elimination of bacterial pathogens\textsuperscript{27–30}. However, they are also expressed in a number of cell types in the brain such as microglia, astrocytes and some specific subsets of neurons\textsuperscript{31–35}. Several independent lines of evidence support a significant contribution of FPRs to the pathological progression of AD. First, FPRs are highly upregulated in reactive glial cells at the site of senile plaques in human AD patients\textsuperscript{36}. Second Aβ\textsubscript{1–42} induced a decrease of cAMP and an induction of ERK phosphorylation in rat microglia and astrocytes, which was inhibited by the synthetic FPR antagonist WRW4\textsuperscript{35,37}. Third, FPR2 dependent recognition of Aβ\textsubscript{1–42} led to the induction of oxidative stress, the release of pro-inflammatory cytokines, and chemotaxis of neutrophils and murine glial cells\textsuperscript{36,38–41}. Fourth, in vitro expression studies demonstrated that Aβ\textsubscript{1–42} can induce calcium mobilization and ERK signaling in cell lines that were transfected with human and murine FPR\textsubscript{2}\textsuperscript{35,38,42}. In addition, FPRs mediate the intracellular uptake of Aβ\textsubscript{1–42} in primary murine glia and in in vitro FPR expression systems\textsuperscript{37,43–45}. Last but not least, treatment with the FPR antagonist Boc2 significantly ameliorated typical symptoms of AD such as cognitive impairment, decreased neuronal density, and Aβ plaque accumulation in an AD mouse model\textsuperscript{46}. Despite these promising results, several key aspects of the interactions between Aβ and the different FPR variants are still insufficiently understood. For example, most studies on the interaction of Aβ with FPRs so far nearly exclusively focused on the role of FPR2. However, the human (h) gene family comprises the three members, hFPR1, hFPR2 and hFPR3. Thus, the potential of the other variants to interact with Aβ has not been extensively examined. Next, the murine (m) gene family comprises seven family members: mFpr1, mFpr2 mFpr3, mFpr-rs3, mFpr-rs4, mFrp-rs6, mFpr-rs7\textsuperscript{47}. The only mouse gene that has a clear genetic orthologue inside the human gene family is mFpr1, which has a common
ancestor with hFPR1\textsuperscript{48,49}. All others are paralogs, which evolved independently after the split of the human and mouse species\textsuperscript{47}, which makes it questionable to which extent data from mouse models can be transferred to humans. Finally, the interactions of FPRs with Aβ were nearly exclusively studied for Aβ\textsubscript{1-42}, while data on the responses to other naturally occurring Aβ variants are lacking.

To address these questions, we used \textit{in vitro} expression of human and mouse FPRs in HEK293T cells in combination with high-throughput measurements of intracellular calcium mobilization to systematically investigate the interactions between different Aβ variants with all human receptors and to compare their response with those of the relevant mouse receptors. In summary, these data provide the first clear evidence for a contribution of FPR1 and FPR3 to Aβ detection and identity N-abridged Aβ fragments as a previously unknown potent group of activators for FPRs. Our results reveal that in addition to mFpr2 and hFPR2 also mFpr1, hFPR1, and hFPR3 are capable to interact with Aβ\textsubscript{1-42}. Next, we can show that the solvent composition and manufacturer of the peptides critically influence the activation of FPRs by Aβ\textsubscript{1-42}. These variations likely depend on a different structural composition of Aβ from different sources. Furthermore, we demonstrate that human and mouse FPRs are also activated by Aβ\textsubscript{1-40} and N-terminally abridged variants such as Aβ\textsubscript{11-40} and Aβ\textsubscript{17-40}. Noteworthy, FPR1 is able to detect these peptides with up to 30-times higher sensitivity than Aβ\textsubscript{1-42}, and that these peptides induce calcium flux and chemotaxis in glial U87 cells that is likely mediated by hFPR1.

\textbf{Results}

\textit{Activation of formyl peptide receptors by amyloid beta is not restricted to FPR2.}

We first investigated the ability of the human receptors hFPR1, hFPR2, and hFPR3 and their mouse counterparts’ mFpr1, mFpr2, and mFpr3 to detect Aβ. The further four existing mouse receptors mFpr-rs3, mFpr-rs4, mFpr-rs6, mFpr-rs7 were excluded from the analysis because they are not expressed in the brain\textsuperscript{48}, and likely don’t mediate classical immune functions\textsuperscript{50}, but are responsible for the detection of yet largely unknown olfactory cues\textsuperscript{50,51}. We first monitored changes in intracellular calcium levels of HEK293T cells transiently transfected with one of the different FPRs after application of Aβ\textsubscript{1-42}. Consistent with previous reports\textsuperscript{36,38}, we observed that Aβ\textsubscript{1-42} induced Ca\textsuperscript{2+} flux at low micromolar concentrations through mouse and human FPR2 (Fig. 1A). Surprisingly, we also observed a similar or even higher amount of activation of mFpr1, hFPR1 and hFPR3, whereas mFpr3 and mock-transfected negative controls did not respond. These data suggest that in addition to hFPR2, all other human FPRs are also able to respond to Aβ\textsubscript{1-42}. Noteworthy, subsequent concentration response tests showed that mouse and human FPR1 were even more sensitive and had a higher signal amplitude than any other receptor (Fig. 1B). Next, we also noticed some important differences between murine and human FPRs. First, we found that mFpr1 responded with an approximately tenfold higher sensitivity to Aβ\textsubscript{1-42} than
mFPR2, whereas the responses of human FPR1 were just slightly better than that of hFPR2. This raises the possibility that mFpr1 might be more relevant for the physiological responses to Aβ1-42 in mouse models than its human orthologue. Second, we found an activation of human FPR3 by Aβ1-42 but did not detect a corresponding signal of its mouse counterpart. In summary, the observed species dependent variations argue for a diverging importance of the different FPR paralogues for Aβ detection and signal transduction in between humans and mice, which should be further examined.

Solvent and supplier variations can critically influence FPR responses to Aβ. A detailed comparison of our results with other publications revealed some inconsistencies in the current literature. While our observation that Aβ1-42 induces Ca²⁺ flux through human and mouse FPR2 in micro molar concentrations is in accordance with all previous studies³⁸,⁴², our study is the first to report an activation of hFPR3. Next, our notion that mouse and human FPR1 are activated by Aβ1-42 is in line with the results reported by Le et al and Slowik et al.³⁵,⁴². However, in these studies the hFPR1 responses were far less pronounced and a study by Tiffany and colleagues even failed to detect any activation of mouse and human FPR1. A careful comparison of the methods used in these and other reports for possible explanations revealed three major potential sources. First, researchers frequently use DMSO to dissolve Aβ1-42, which can be critical in the case of FPRs because DMSO is an agonist for FPR1 and FPR2 and may therefore affect the Aβ1-42-evoked signals⁵⁰. Second, varying types of physiological assay buffers and solvents were used, which might be critical in the case of Aβ1-42 because this peptide tends to form diverse kinds of aggregates in different assay buffers²⁰,⁵²,⁵³. Third, Aβ1-42 was obtained from different sources, which raises the possibility that variations in the production processes may have influenced the peptide aggregation status. We therefore decided to carefully investigate the influence of these factors on the FPR responses. To examine the impact of peptide synthesis on the receptor responses we ordered four additional Aβ1-42 peptides from three additional sources (Anaspec, Sigma-Aldrich, and Synpeptide) and compared them to the peptide that we obtained from our initial supplier Peptides&Elephants (P&E). A second peptide from Anaspec that was Hexafluoroisopropanol (HFIP) treated was used as positive control for aggregation because this peptide is known to readily form aggregates. Using a thioflavine T (ThT) assay (Fig. 2A) we first compared the aggregation status 10 minutes after dissolving the peptides with their kinetics over the course of the next two hours because we estimated that this would be the maximal time span to perform our calcium imaging experiments. We already observed clear differences between the four supposedly identical peptides in the 10 minutes after dissolving them in our assay buffer. In comparison to the peptide from P&E, Aβ1-42 from Synpeptide and Sigma-Aldrich showed an approximately 30 % to 50 % lower aggregate content whereas, the non-HFIP treated peptide from Anaspec was approximately 30% more pre-aggregated. The kinetics in the first 2 hours revealed that the aggregation control with the HFIP-treated peptide showed the expected increase, whereas the status of Aβ1-42 from Sigma-Aldrich, and Synpeptide did
not drastically change. The non-HFIP treated Anaspec peptide even showed a reduction of fluorescence. In transmission electron microscopy (Supporting Fig. S1A), Aβ_{1-42} from P&E, Synpeptide, and the HFIP-treated Anaspec peptide displayed a somewhat similar amorphous morphology, while the non-HFIP treated Anaspec peptide seemed to directly form long fibers, which may explain the high starting fluorescence in our ThT assay. Together, these results indicate a considerable amount of heterogeneity in the secondary and tertiary structure of peptides from different sources. In line with this, Ca^{2+} flux measurements also revealed clear differences in the response pattern of mouse and human FPRs to these peptides (Fig. 2B & 2C and Supporting Fig. S1B). Aβ_{1-42} from Sigma-Aldrich elicited a similar response pattern as that of P&E. However, it failed to activate hFPR1. When using Aβ_{1-42} from Synpeptide the responses of hFPR1 and hFPR2 were lost. The non-HFIP treated peptide from Anaspec potently activated mFpr1 but failed to activate any other mouse or human FPR, whereas the HFIP treated version weakly activated hFPR3, mFpr1 and mFpr2. Thus, variations in the manufacturing process are critical factors that are capable to strongly affect the activation pattern of FPRs. To evaluate the secondary structure of our peptides, we next performed Circular Dichroism (CD) spectroscopy on Aβ_{1-42} peptides from P&E, Synpeptide, and the two variants from Anaspec, all dissolved in the assay buffer C1 (Fig. 2D and supporting Fig. 1C). All tested peptides showed similar proportions of α-helix and β-sheet, but differed in the amount of β-turns and unstructured sequences. In comparison with the two different Anaspec peptides that elicited only relatively weak calcium responses, the Aβ_{1-42} peptides from P&E and Synpeptide, which were able to more robustly activate FPRs, displayed significantly elevated β-turn proportions. This suggests that increased proportions of β-turn conformation in Aβ peptides may improve their interaction with FPRs. We next examined the influence of different assay buffers on FPR signals. To this end, we first compared the FPR responses to Aβ_{1-42} from P&E dissolved in our assay buffer C1 with those of the two frequently used buffers HBSS and Tris-NaCl (Fig. 2E). We detected the most robust activation of human and mouse FPRs in C1. The use of Tris-NaCl lead to an approximately 50% and 20% reduction of the signal amplitude of hFPR1- and hFPR2-based response respectively, but did not significantly alter the responses of hFPR3. Use of HBSS, by contrast, resulted in a complete loss of nearly all FPR responses. Interestingly, this was clearly correlated with a strong reduction in the capability to form aggregates (Fig. 2F). Furthermore, we even observed that a longer storage of the dissolved peptides in the freezer can affect the response pattern (Supporting Fig. S2). Next, we carefully examined the effect of the commonly used solvent DMSO on the Aβ_{1-42} response pattern of the individual FPRs (Supporting Fig. S3). Again, we observed a number of subtle changes in the FPR response patterns that were difficult to predict because they largely depended on a specific combination of receptor subtype and peptide source. For example, DMSO diminished the hFPR1 response to Aβ_{1-42} from P&E by 40%, whereas it increased the response of hFPR3 by 32%. Despite these variations, our data clearly demonstrate that mFpr1 and hFPR3 were activated
under almost all conditions. However, the overall activation pattern of FPRs showed considerable alterations that depend on manufacturing source, assay buffer conditions, storage time and the pretreatment of Aβ_{1-42} with different solvents. Thus, there is a clear need for standardization and careful description of all experimental procedures.

**N-abridged Aβ-fragments are potent activators of mouse and human FPR1.** Under *in vivo* conditions not only Aβ_{1-42} but several other fragments such as Aβ_{1-40}, Aβ_{11-40} and Aβ_{17-40} are also generated during the cleavage of APP and its subsequent processing^{12,54,55}. Thus, it is conceivable that FPRs can also interact with some of these fragments. To test this hypothesis, we investigated the response of FPRs to the commonly found Aβ_{1-40} the N-abridged variants Aβ_{11-40} and Aβ_{17-40}, and the C-terminally abridged fragments Aβ_{1-10} and Aβ_{1-16} (Fig. 3A). We observed that longer C-terminal deletions tend to be detrimental for the activation of FPRs because fragments such as Aβ_{1-10} and Aβ_{1-16} failed to induce any Ca^{2+} mobilization. In sharp contrast, the two tested N-abridged variants Aβ_{11-40} and Aβ_{17-40}, elicited responses on all FPRs that we could previously activate with the full-length Aβ_{1-42}. Unlike the responses to the full-length Aβ_{1-42}, the FPR responses to these shorter fragments seemed to be less prone to manufacturer-dependent variations (Supporting Fig. S4). Surprisingly, N-terminal deletions tended to strongly improve the interaction with FPR1. Concentration response curves revealed that mouse and human FPR1 could detect both N-abridged fragments with more than 10fold higher sensitivity than the full-length peptide Aβ_{1-42}, (Fig. 3B and 3C). Mouse and human FPR2 only responded to micro molar concentrations of these peptides (Fig. 3B). The observation that Aβ_{11-40} and Aβ_{17-40} can already induce an activation of mouse and human FPR1 at 30 to 100 nano molar concentration raises the possibility that the detection of these short fragments through FPR1 might be even more relevant for the physiological response than the activation of FPR2 by longer Aβ at micro molar concentration.

**N-abridged Aβ variants are potent activators for a human glia cell line.** So far, our study focused on the characterization of the FPR pharmacology in an *in vitro* over-expression-based system. Despite the strength of this system to dissect responses of the individual FPRs to Aβ we cannot exclude that differences in the signal transduction system of HEK293T cells or the overexpression of the receptors may affect our pharmacological results. We therefore sought to validate our results in a more biologically relevant setting. To this end, we examined the responses elicited by Aβ_{11-40} and Aβ_{17-40} in U87 cells (Fig. 4) that are a commonly used as a human glial cell model and are known for their natural expression of FPRs^{56,57}. We first used RT-PCR and RT-qPCR to examine the expression of the individual FPRs in these cells. Our data revealed that these cells show high mRNA levels of hFPR1 but only a very modest expression of hFPR2 and hFPR3 (Fig. 5A and Supporting Fig. S6A and S7). Immunocytochemistry with receptor subtype specific antibodies (Fig. 5B and Supporting Fig. S5) confirmed a robust expression of hFPR1 and lower amounts of hFPR2. Interestingly, hFPR3 showed a
relatively abundant protein amount despite low amounts of mRNA which is consistent with similar findings for mFpr3 expression in mouse immune cells.58

Calcium imaging experiments revealed that a stimulation with Aβ11-40 and Aβ17-40 resulted in a pronounced activation of U87 cells (Fig. 4A and 4B) that were closely similar to the typical FPR time kinetics observed in the transfected HEK cells (Fig. 1). In chemotaxis experiments only Aβ11-40 but not Aβ17-40 induced migration of these cells (Fig. 4C and 4D). Both, the calcium signals and the chemotaxis were FPR dependent because a co-application of tBoc2, which is a competitive antagonist of FPR1 and FPR2 but does not inhibit FPR3 (Supporting Fig. S6B), completely abolished the responses. To address the question which FPR exactly is responsible for the observed results, we first compared the U87 concentration response curves of Aβ11-40 and Aβ17-40 with those of FPR-transfected HEK293T cells. We found them to be remarkably similar to hFPR1 (Fig. 5C). Next, we used receptor subtype specific activators to tests which receptor can trigger a calcium signal. To this end, we challenged U87 cells and hFPR-transfected HEK293T cells with two bacterial signal peptides SP6 and SP4 at concentrations where they would only activate either hFPR1 or hFPR2, respectively50. Of note, both compounds would not activate hFPR3 (Supporting Fig. S6C). In line with our previous notion that responses to the N-terminally abridged Aβ variants likely depend on hFPR1, the U87 cells only responded to the hFPR1 specific SP6 stimulus with a calcium signal, but not to the hFPR2 specific SP4 stimulus (Fig. 5D and Supporting Fig. S6C). Next, we performed cross desensitization experiments, which showed that responses of U87 cells towards Aβ17-40 were absent after pre-application of the FPR1 specific agonist SP6 but were not influenced by pre-application of the FPR2 specific agonist SP4 (Fig. 5E). Finally, in the chemotaxis assay U87 cells only migrated towards the FPR1 specific SP6 but not towards the FPR2 specific SP4 (Fig. 5F). Taken together these experiments strongly argue that the sensitive calcium and chemotaxis responses of U87 to N-terminally abridged Aβ variants depend on an activation of hFPR1.

Discussion

In summary, our study provides a systematic overview over the capability of different mouse and human FPRs to interact with Aβ in different buffer systems. To our knowledge, we are the first to report an activation of hFPR3 by Aβ and the first to identify N-terminally abridged Aβ fragments as activators of FPRs. Moreover, our data show that hFPR1 and its mouse orthologue mFpr1 can recognize N-terminally abridged fragments such as Aβ11-40 and Aβ17-40 at drastically lower concentrations than longer peptides such as Aβ1-40 and Aβ1-42, which raises the possibility that FPR1 might be more relevant for the pro-inflammatory physiological responses to Aβ than FPR2. Interestingly, several independent lines of evidence from literature are highly consistent with this hypothesis. First, FPR1 is already expressed at the resting state of glial cells and is further upregulated during their transformation into the reactive
state. Next, mFpr1 is highly upregulated in the cortex and hippocampus of transgenic APP/PS1 mice, which are a commonly used AD animal model. Moreover, FPR1 has been shown to mediate typical pro-inflammatory effects in glial cells that are also commonly observed in AD such as generation of oxidative stress, release of inflammatory cytokines and chemokines and the induction of cell migration whereas several reports suggest an anti-inflammatory role of FPR2. Next, treatment of APP/PS1 mice with the competitive FPR antagonist tBoc2 that preferentially binds to FPR1 leads to reduced microglia reactivity, decreased neuronal pathology and improved cognitive performance. Taken together, these findings strongly support our notion that FPR1 and other FPR variants contributes to neuroinflammation of AD through their ability to sense different Aβ variants. Of note, this contribution might be rather complex because we here observed that abridged variants sometimes trigger partially different signaling pathways (e.g. calcium signaling only for Aβ17-40 or calcium signaling and migration for Aβ11-40) likely through exclusive activation of the identical receptor. Next, a given Aβ peptide will show a concentration-dependent interaction with different FPR receptors. Finally, other pro- and anti-inflammatory FPR ligands such as Annexin A1, Lipoxin A4, Resolvin or mitochondrial peptides may further modulate these signals depending on their local concentration. However, the observed 10 to 30fold higher affinity of FPR1 to N-abridged fragments insinuates that such fragments might be of high pathological relevance for AD if they occur under physiological conditions in sufficient concentrations. Indeed several studies suggest that Aβ11-40/42 is present in the CSF at similar concentrations as Aβ1-42 and is furthermore an integral part of senile plaques. Aβ17-40/42 is thought to occur at similar rates as Aβ1-42 and Aβ11-40/42, however its exact quantification is difficult and therefore often disregarded. In accordance with our data, both Aβ11-40 and Aβ17-40 were shown to activate glial cells, which is associated with the neurotoxic effects seen in AD. Moreover our observation that Aβ11-40 but not Aβ17-40 induced chemotaxis of U87 cells is in line with previous results that show that Aβ17-40/42 and Aβ17-43 only lead to a partial activation of human glial cells. Thus, to the best of our knowledge our data are consistent with a model in which recognition of these N-abridged Aβ variants via FPR1 could thus occur under physiologically conditions. Finally, our results clearly demonstrate that the precise FPR responses towards different Aβ variants are highly subjectable to solvent- and manufacturer-dependent effects. Factors such as the secondary structure, 3D conformation, and aggregation therefore seem to be of high importance for the precise activation pattern and different receptor subtypes seem to prefer different peptide structures or exposed surfaces. Multiple previous studies have already shown that different buffer systems and solvents can critically influence properties of Aβ such as its aggregation kinetics and the structure of its aggregates. For example, Szczepanik and colleagues observed differences in Aβ-dependent cytokine release depending on the solvent of their peptides. We assume that variations within our measurements occur due to formation of aggregates and variations in the secondary structure as
indicated by our CD and TEM measurements. In accordance with this idea, in previous studies both monocytes and microglia lost FPR-dependent signaling with progressing aggregation of Aβ_{1-42}\textsuperscript{42,69}. Of note, it is conceivable that chemical modifications of the peptide e.g. oxidation of methionine at position 35, racemization or isomerization may contribute to the functional differences that we observed for an identical peptide in different buffer systems. This needs to be further examined. However, our data also show that it is necessary to develop a generally accepted standardized protocol for the investigation of FPR-related effects on Aβ peptides. In many fields the use of similar assay conditions has thus improved the reproducibility and quality of data\textsuperscript{20}. Based on our current results we would like to recommend the following protocol (see Fig. 6). We propose to validate any observed Aβ effects with a peptide from at second supplier. Next, we would like to encourage researcher to also include negative results with peptides from other sources. All experiments should be performed using single use aliquots from a freshly dissolved frozen stock. Next, any stocks that are stored should be examined for “aging” effects. We also suggest the use of low complexity buffer systems such as our C1 buffer and avoid co-solvents such as DMSO if possible. In case DMSO or other co-solvents are used their effects on aggregation and physiological response need to be controlled and reported. All experiments should be performed within the same standardized time in order to have a similar amount of aggregation. Finally, we suggest to always include at least some data on the aggregation status and time kinetics of the peptides in a given buffer system, which can be easily done with simple inexpensive methods such as ThT assays. Moreover, we would like encourage a very detailed description of all experimental conditions that may affect the aggregation as Supporting material. Especially the peptide source including lot numbers, purity, used solvents, precise assay buffer composition, pH, incubation time, storage time, storage conditions, and final concentration of co-solvents in an assay should be reported because this all can critically influence the results.

**Experimental procedures**

*Cloning of Human and Murine FPR Genes*

Human hFPR1, hFPR2 and hFPR3 and murine mFpr1, mFpr2 and mFpr3 were amplified from genomic human or C57BL/6J murine DNA respectively and sub-cloned into pcDNA3.1\textsuperscript{(+)} (Invitrogen) as previously described\textsuperscript{50}.

*Ligands and chemicals*

Purity of all peptides was at least >95%. Details on manufacturer, Catalogue numbers, lot numbers, exact purity of all peptides, solvents, and storage conditions are given in supplementary table 1. The amino acid composition of all Aβ peptides corresponded to the human wild type sequence. Aβ_{1-42}
peptides were purchased from Anaspec/MoBiTec, Peptides&Elephants and Sigma-Aldrich or synthesized by Synpeptide. All Aβ1-42 peptides were dissolved at 30 µM in C1 assay buffer (130 mM NaCl, 10 mM HEPES, 5 mM KCl, 2 mM CaCl2, pH 7.2, all purchased from Carl Roth). To fully dissolve the peptides in C1, they were placed in an ultrasonic bath for usually 5-10 min at room temperature until all precipitates had vanished. Peptides dissolved in an aqueous solution were then aliquoted for single-use, immediately stored at -20°C and used up within 6 weeks because longer storage may affect the results. If not otherwise stated experiments were performed with Aβ1-42 dissolved in C1. For selected experiments (Suplementary table S1) where we investigated the effects of different solvents Aβ1-42 from Anaspec/MoBiTec, P&E and Synpeptide were dissolved at 5 mM in DMSO (Sigma-Aldrich), while Aβ1-42 from P&E was additionally dissolved at 30 µM in HBSS (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 0.4 mM Magnesium Sulfate Heptahydrate, 0.5 mM Magnesium Chloride Hexahydrate, 0.3 mM Sodium Phosphate Dihydrate, 0.4 mM Potassium Phosphate, 4 mM Sodium bicarbonate, pH 7.2, all purchased from Carl Roth) or Tris-NaCl (50 mM Tris, 150 mM NaCl, pH 7.2). Peptides in HBSS or Tris-NaCl were dissolved in an ultrasonic bath as described above. HFIP-pretreated Aβ1-42 (in the main text referred to as Anaspec-HFIP) was purchased as AggreSure Aβ1-42 from Anaspec/MoBiTec and dissolved at 5 mM in DMSO for calcium imaging experiments or in Tris-NaCl (50 mM Tris, 150 mM NaCl, pH 7.2) for aggregation assays. Aβ1-40, Aβ1-10 and Aβ1-16 were purchased from Anaspec/MoBiTec. The Aβ17-40 peptide used throughout the main text was purchased from Anaspec/MoBiTec and validated with Aβ17-40 peptides from Sigma-Aldrich and Synpeptide. Aβ11-40 was obtained from Peptides&Elephants. Aβ1-10 and Aβ1-16 were dissolved in C1 as a 1 mM stock solution and Aβ1-40 at 30 µM as described above. Due to their high hydrophobicity Aβ11-40 and Aβ17-40 were dissolved in DMSO at 5 mM. Tert-Boc-FLFL (tBoc2) was purchased from Bachem and dissolved in DMSO at 30 mM. f-MLFYFS (Psy-SP6) and f-MAMKKL (Sal-SP4) were obtained from VCPBIO and dissolved at 10 mM in DMSO (Psy-SP6) or at 1 mM in C1 (SP4) respectively. WKWMVm-NH2 and WKWMVm-CHO were purchased from Proteogenix and VCPBIO respectively and dissolved as 1 mM stock solutions in C1. fMLF was purchased from Sigma-Aldrich and also dissolved at 1 mM in C1. All peptides were thawed at room temperature approximately 1 h before each functional experiment, as this time was needed to prepare the compound plates for each measurement.

**Cell Culture and Transient Transfections**

HEK293T cells (ATCC) were cultured in Dulbecco’s Modified Eagle’s medium (DMEM, Biowest) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, Pan Biotech), 1 unit/ml penicillin-streptomycin (Biowest) and 2mM L-glutamine (Biowest) until 80% confluence. For transfection, approximately 2,000 cells were seeded in each well of poly-D-lysine-coated (PDL) (10 µg/ml in PBS, Sigma) black optical 96-well µCLEAR-plates (Greiner Bio-One). Cells were transfected after 24h using jetPEI (Polyplus-transfection SA) with 0.125 µg of DNA plasmids encoding the respective receptors and
equal amounts of a plasmid encoding the G protein subunit \( \alpha_{16} \) since this subunit is needed to trigger FPR dependent calcium signals in HEK293 cells \(^{50,70}\). The medium of transfected cells was changed 24h after transfection. For mock transfections the proportion of the FPR containing plasmids were substituted by an empty pcDNA3.1 vector.

U87 MG cells (CLS) were cultured in Minimum Essential medium (MEM, Biowest) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, Pan Biotech), 1 unit/ml penicillin-streptomycin (Biowest), 2mM L-glutamine (Biowest) and 1 unit/ml non-essential amino acids (NEAA, Biowest) until 50-60% confluence. For calcium imaging and immunostaining, on average 2,500 cells were seeded in each well of Poly-D-lysine-coated (10 µg/ml in PBS, Sigma) black optical 96-well µCLEAR-plates (Greiner Bio-One).

**Calcium Imaging**

Cell population responses of transfected HEK293T cells and U87 cells were recorded using a Flexstation III microplate reader (Molecular Devices). Briefly, cells were incubated with 2 µM Calbryte™ 520 AM (AAC Bioquest) for 2h at room temperature in C1 assay buffer with 5 mM glucose (Carl Roth). Before each experiment, cells were rinsed three times with C1. Experiments with HEK293T cells were conducted 48h after transfection, while experiments with U87 cells were performed 24h after seeding. Acquisition of baseline fluorescence was performed for 25 s before ligand application and cell population response was measured for 125 s after application. Responsiveness of cells was controlled the appropriate buffer and solvent controls and with 10 µM WKWMVm-NH\(_2\) or WKWMVm-CHO as those ligands are potent activators of all three human and mouse FPRs\(^{50}\).

**Chemotaxis Assays**

Cell migration assays were performed and analyzed with an IncuCyte S3 Life Cell Imaging System (EssenBio Science). Briefly, U87 cells were harvested and resuspended in Minimum Essential medium (MEM, Biowest) supplemented with 0.5% (v/v) heat-inactivated fetal calf serum (FCS, Pan Biotech), 1 unit/ml penicillin-streptomycin (Biowest), 2mM L-glutamine (Biowest) and 1 unit/ml non-essential amino acids (NEAA, Biowest). Approximately 1000 cells were placed into the top chamber of each well of a 96-well ClearView Plate (EssenBioscience). Chemoattractants were mixed in 200 µl medium and then placed in the corresponding bottom chambers. Image acquisition was performed hourly for 24 h on both sides of a membrane separating the top and bottom chambers. Subsequent analysis was performed using the IncuCyte S3 software.

**Immunostaining**

Approximately 2,000 HEK293T cells or 3,500 U87 cells were seeded in each well of a PDL-coated black optical 96-well µCLEAR-plate (Greiner Bio-One). Transient transfection of HEK293T cells was
performed as described above. 24h after transfection cells were fixated with 4% [v/v] methanol-free paraformaldehyde (Polyscience Inc.) in PBS for 30 min at RT and afterwards rinsed with PBS. After blocking with 5% [v/v] FCS in PBS for 30 min at RT, primary antibodies diluted in blocking solution were applied to the cells and incubated over night at 4° C. Hereby, monoclonal antibodies for hFPR1 (R&D Systems, MAB3744, 1 µg/ml), hFPR2 (Santa Cruz Biotechnology, sc-57141, 0.2 µg/ml) and hFPR3 (R&D Systems, MAB3896, 1 µg/ml) were used. Cells were rinsed three times with PBS and subsequently treated with 2 µg/ml polyclonal alpaca anti-mouse antibody conjugated with Alexa Fluor 568 (Invitrogen) and 2 µM Hoechst 33342 (Thermo Fisher) for 60 min at RT. Image acquisition was performed with a Molecular Devices ImageXpress Micro confocal microscope and analyzed using MetaXpress software (Molecular Devices).

**PCR Analysis**

Total RNA was isolated using AnalytikJena innuPREP RNA Kit according to the manufacturer’s protocol. Reverse transcription was carried out using 30 ng of total RNA and Superscript II Reverse Transcriptase (ThermoFisher Scientific). Initial RT-PCR experiments were performed using DreamTaq DNA polymerase (ThermoFisher Scientific) with primers (Sigma-Aldrich) comprising the full coding region with cDNA obtained from 0.3 ng total RNA in a total reaction volume of 20 µl. PCR conditions were: 95ºC for 3 min, 35 cycles at 95ºC for 30s, 64ºC for 30s and 72ºC for 60s, followed by a final extension of 72°C for 10 minutes. qPCRs were performed with the Biozym Blue S’Green qPCR Kit according to the MIQE guidelines. RT-qPCR reactions were carried out with cDNA obtained from 0.15 ng total RNA as duplicates in 20 µl total reaction volume. PCR conditions were: 95ºC for 3 min, 32 cycles at 95ºC for 5s, 66ºC for 10s and 66ºC for 10s. Representative samples of all PCR products were assessed by gel electrophoresis and sequencing for their quality. Absolute quantification of DNA copies was calculated according to the specific standard curves supplemented in Supplementary Fig S6. For qPCR for calibration curves a dilution series of a sequenced and purified PCR product in 2 ng/µl yeast tRNA (Sigma-Aldrich) was used. In additional relative quantifications for a house keeping gene were performed using GAPDH. All PCRs were performed in a TOPical T-Gradient thermocycler (Biometra). Primer sequences are given in Supporting Table S2.

**Thioflavin T (ThT) aggregation assay**

For the experiments in C1 buffer Aβ$_{1-42}$ peptides were dissolved as 30 µM stock solutions. For experiments with different buffers, Aβ$_{1-42}$ from P&E was dissolved either at 5 mM in DMSO or at 30 µM in HBSS or Tris-NaCl as described above. HFIP-treated Aβ$_{1-42}$ was dissolved at 5 mM in DMSO and measured in C1 with a final concentration of 0.2% [v/v] DMSO. ThT (Sigma-Aldrich) stock solutions were dissolved at 1 mM in the respective buffer, sterile filtered with 0.2 µM pore membranes and stored at -20° C. Aβ peptides were combined with ThT stocks to produce working solutions with final
concentrations of 22.5 µM Aβ1-42 and 250 µM ThT in the respective buffer. Fluorescence measurements were carried out as 100 µl triplicates in black optical 384-well µCLEAR plates (Greiner Bio-One) at 37°C with excitation at 440 nm and emission at 484 nm in a FlexStation III microplate reader within 15 min after thawing of the peptides. Signals were measured in intervals of 60 s for 120 min and were shaken for 3 s before each read.

**Binding Assay**

Approximately 2,000 HEK293T cells were seeded in each well of a PDL-coated black optical 96-well plate and transfected as described above. 48h after transfection, cells were treated with 1 µM of FITC-labeled WKVVm-NH₂ and 20 µM Hoechst 33342 diluted in DMEM and incubated for 30 min at 37°C and 5% CO₂. Cells were then rinsed ten times with C1. Image acquisition was performed with a Molecular Devices ImageXpress Micro confocal microscope and analyzed using MetaXpress software (Molecular Devices).

**Circular Dichroism Spectroscopy (CD)**

30 µM of Aβ1-42 dissolved in C1 were sonicated for 10 minutes before CD measurement. CD spectra were recorded (JASCO J-1500 spectrometer) in a 1 mm High Precision Cell (Hellma Analytics). Data were processed in Spectra Analysis by JASCO and were plotted by Origin.

**Transmission Electron Microscopy (TEM)**

30 µM of Aβ1-42 peptide solution was first incubated for 24 h and then deposited on Formvar/carbon-film coated copper grids (Plano GmbH). Samples were then stained with 4% uranyl acetate. Subsequently, TEM images were acquired (JEOL 1400 Transmission Electron Microscope) and then processed in ImageJ.

**Statistical methodology**

Statistical significances were calculated using either the unpaired student’s t-test with assumption of Gaussian distribution or One-way ANOVA with Dunnett’s or Tukey’s multiple comparisons post hoc analysis. Calculations were performed using GraphPad Prism 9.2.

**Data Availability**

The authors confirm that the data supporting the findings of this study are available within the article and its supporting information.

**Supporting information**

This article contains supporting information.
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Author contributions

L.B. performed most experiments, analyzed data, prepared all figures and contributed to writing the manuscript. Z.T. performed qPCR experiments, analyzed data and contributed to figure design and the writing process. Y.T. performed CD and TEM measurements, analyzed data and contributed to figure design and the writing process. V.T.T.N. performed initial ThT experiments and helped in establishing the method. Q.L. performed TEM measurements. C.V.S. designed CD and TEM experiments and provided theoretical insights. K.E. helped to design the initial ThT experiments, provided theoretical insights and participated in writing the manuscript. B.B. designed the study and wrote the manuscript.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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**Figure legends**

**FIGURE 1.** Aβ1-42 activates all human FPRs. A, representative Ca^{2+} traces of HEK293T cells transiently transfected with FPR plasmids or an empty vector (mock) after stimulation with Aβ1-42 obtained from P&E. B, mean peak Ca^{2+} responses of human (red) and mouse (blue) FPRs upon stimulation with different concentrations of Aβ1-42. Buffer (grey) denotes responses to the assay buffer without Aβ1-42. Bars represent mean values of three independent experiments (n=3) carried out as technical duplicates (N=2). All Error bars, S.D.; One-way ANOVA test, Dunnett post hoc test; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ns, no significance.

**FIGURE 2.** Manufacturer- and solvent- effects on FPR activation by Aβ1-42. A, Left: Schematic depiction of ThT aggregation assay. Right: Mean fluorescence of 22.5 µM Aβ1-42 in C1 buffers from different manufacturers in a ThT aggregation assay during the first 10 minutes (clear bars) versus fluorescence after 120 min (striped bars). Buffer refers to ThT fluorescence without addition of peptides (grey bars). All n=3, N=3, except for Sigma and Anaspec with n=2, N=3; One-way ANOVA test, Dunnett post hoc test. B, mean Ca^{2+} peak responses of human (red) or mouse (blue) FPRs to 10 µM of Aβ1-42 peptides obtained from Peptides & Elephants (P&E) and Synpeptide; n=3, N=2, One-way ANOVA test, Dunnett post hoc test in comparison to respective buffer controls C, heat map of mean Ca^{2+} responses of FPRs elicited by Aβ1-42 peptides obtained from five different manufacturers. The scale ranges from white (no response) to deep orange (ΔF/F0 ≥ 0.4). Responses are shown in supporting figure S1. D, secondary structure composition of four Aβ1-42 peptides analyzed by CD spectroscopy; n=3, N=1; One-way ANOVA test, Tukey post hoc test. E, mean Ca^{2+} peak responses of cells transfected with human FPRs (red) or mock (grey) towards 5 µM Aβ1-42 dissolved in the respective buffers; n=3, N=1, One-way ANOVA test, Dunnett post hoc test. F, Comparison of ThT fluorescence of Aβ1-42 (P&E) dissolved in either C1, DMSO, Tris-NaCl or HBSS. All assays were performed in the respective buffers. For experiments with peptides pre-dissolved in DMSO, assays were conducted in C1 with a final concentration of DMSO: 0.4% (V/V). One-way ANOVA test, Dunnett post hoc test. All Error bars, S.D.; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ns, no significance.
FIGURE 3 Naturally occurring N-abridged Aβ fragments activate FPR1 tenfold better than Aβ1-42. A, Comparison of mean Ca²⁺ peak responses of human (red) or mouse (blue) FPRs to a stimulation with 10 µM Aβ1-40 and Aβ1-42, or 5 µM of the natural occurring N-abridged variants Aβ11-40 and Aβ17-40 or with 10 µM of the C-abridged variants Aβ1-10 and Aβ1-16. Colored bars indicate responses of human (red) or mouse (blue) FPRs, n=3, N=1. B, Concentration response curves of selected variants, n=3, N=1. C, left: scheme indicating the size and location of the different Aβ variants. Right: Table depicting the proposed 3D-structures and thresholds for minimal detectable activation during Ca²⁺ imaging of the responding Aβ variants. All Error bars, S.D. One-way ANOVA test, Dunnett post hoc test; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ns, no significance
FIGURE 4  
**N-abridged Aβ peptides induce hFPR1-dependent responses in glial U87 cells.**  
**A**, Mean Ca\(^{2+}\) peak responses of U87 cells after stimulation with different concentrations of Aβ\(_{11-40}\) or Aβ\(_{17-40}\). Striped bars indicate the response towards 10 µM of the positive control WKWVm-NH\(_2\), light grey and dark grey bars indicate negative controls; n=3, N=1.  
**B**, Comparison of the Ca\(^{2+}\) responses upon stimulation with either 5 µM Aβ\(_{11-40}\) or Aβ\(_{17-40}\) alone (green bars) or in the presence of 10 µM of the competitive FPR-antagonist tBoc2 (**black bars**); n=3, N=1.  
**C**, dose-dependent chemotaxis of U87 cells upon stimulation with either Aβ\(_{11-40}\) or Aβ\(_{17-40}\). Each bar represents the number of cells that migrated through a porous membrane towards the respective stimuli. **Green bars** indicate migration towards N-abridged fragments, striped bars display migration towards the positive control 1 µM fMLF, light grey bars show migration without stimuli and dark grey bars represent the response to 0.1% DMSO, n=3, N=1.  
**D**, Migration of U87 cells that were either untreated (**green bars**) or treated with 10 µM tBoc2 (**black bars**) towards 1 µM of Aβ\(_{11-40}\) or Aβ\(_{17-40}\); n=3, N=1. All Error bars, S.D. One-way ANOVA test, Dunnett post hoc test for A and C and t-test for B and D; *, p ≤ 0.05; ***, p ≤ 0.01; ns, no significance.

FIGURE 5  
**The activation of U87 cells by of N-abridged Aβ peptides depends on hFPR1.**  
**A**, PCR experiments show that U87 cells contain high mRNA levels of hFPR1 but only low amounts of FPR2 and FPR3. Left: Representative image of gel electrophoresis after RT-PCR with primers for all hFPRs. Right: Quantification of cDNA for all FPRs obtained through RT-qPCR, n=5, N=2. Details on the primer efficiency, specificity and linearity are given in supporting Fig. S6 and S7.  
**B**, left: representative immunocytochemistry staining of U87 cells and transfected HEK293T cells with FPR-subtype specific antibodies (red) and nuclei staining (blue). For visibility brightness and contrast were adjusted for U87 cells and HEK293T cells differently, for absolute intensity comparison pictures with equal settings. n=2, N=2 for U87 cells and n=2, N=2 for HEK293T cells. Evidence for closely similar staining intensities of FPR1 and FPR2 by the used protocols are shown in supporting Fig. S5. One-way ANOVA test, Tukey post hoc test.  
**C**, the concentration dependent Ca\(^{2+}\) responses of HEK293 cells and U87 cells towards the N-abridged fragments Aβ\(_{11-40}\) and Aβ\(_{17-40}\) reveal a clear correlation with the hFPR1 response, n=3, N=1.  
**D**, U87 cells respond to the potent
hFPR1 activator SP6 (10 nM) but not to a potent hFPR2 agonist SP4 (10 nM) in calcium imaging experiments, n=3, N=3. One-way ANOVA test, Dunnett post hoc test. E, Cross-desensitization experiment of U87 cells show that the Ca²⁺ responses towards Aβ17-40 are abolished by the FPR1 activator SP6 but not by the hFPR2 agonist SP4. Representative Ca⁺ traces (left) and mean Ca²⁺ peak responses (right) to a secondary Aβ17-40 stimulus after pre-application of SP4 or SP6 as a first stimulus, n=1, N=3; t-test. F, U87 migrate towards the hFPR1-stimulus SP6 (10 nM) but do not respond to the hFPR2 agonist SP4 (10 nM), n=3, N=1. One-way ANOVA test, Tukey post hoc test. All Error bars, S.D. *, p ≤ 0.05, **, p ≤ 0.01, ***, p ≤ 0.001; ns, no significance.

FIGURE 6 Minimal requirements for the investigation of Aβ interactions with FPRs

Supporting Information

FIGURE S1 Manufacturer-dependent FPR activation by Aβ1-42 A, representative TEM images of Aβ1-42 obtained from either Peptides&Elephants (P&E), Synpeptide, or Anaspec show different types of aggregates. AS-HFIP refers to a commercially available HFIP-treated peptide from Anaspec. Scale bars indicate 0.5 µm. B, mean Ca²⁺ peak responses of FPR-transfected HEK293T cells after stimulation with 10 µM Aβ1-42 obtained from different manufacturers. Colored bars indicate the response of human (red) or mouse (blue) FPRs, n=3, N=2. C, complete CD spectra of all four Aβ1-42 displayed in figure 2. N=3. All Error bars, S.D.; One-way ANOVA test, Dunnett post hoc test; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ns, no significance

FIGURE S2. Alterations of FPR responses to Aβ1-42 after extended storage. Comparison of the Ca²⁺ responses of human (red) or mouse (blue) FPRs to 10 µM Aβ1-42 (Synpeptide) that were either freshly dissolved in the assay buffer C1 (clear bars) or had been stored in C1 at -20° C for 6 months without freeze-thaw cycles (striped bars), n=1, N=2. All Error bars, S.D.; *, p ≤ 0.05; ns, no significance.

FIGURE S3. DMSO influences FPR activation by Aβ1-42. Mean Ca²⁺ peak responses of human (red) or mouse (blue) FPRs to 5 µM of Aβ1-42 peptides obtained from P&E, Synpeptide or Anaspec dissolved in either DMSO (striped bars) or C1 (clear bars), n=3, N=2. All Error bars, S.D.; *, p ≤ 0.05; ns, no significance.
FIGURE S4. Aβ_{17-40} is less susceptible to manufacturer-dependent effects than full length Aβ. Mean Ca^{2+} peak responses of FPR-transfected HEK293T cells to 5 µM of Aβ_{17-40} from three different manufacturers. Clear bars, Aβ_{17-40} from Anaspec. Striped bars, Aβ_{17-40} from Synpeptide. Dotted bars: Aβ_{17-40} from Sigma-Aldrich, n=3, N=1. All Error bars, S.D.; One-way ANOVA test, Dunnett post hoc test; *, p ≤ 0.05; ns, no significance.

FIGURE S5. Validation of immunofluorescence staining with FPR subtype-specific antibodies. Top: Comparison of immunofluorescence staining intensity of FPR-transfected HEK293T and U87 cells with receptor subtype specific antibodies. The hFPR1 (1 µg/ml), hFPR2 (0.2 µg/ml) and hFPR3 (1 µg/ml) antibodies were used. Note that the absolute staining intensities of all hFPRs in HEK293T cells were highly similar, however a strong reduction of hFPR2 staining in U87 cells is observed. No cross reactivity was detected. All images were acquired with the same settings and are displayed with the same illumination, brightness and contrast. Evidence for similar cell surface expression and production rates for hFPR1 and hFPR2 can be found in supporting Figure S8. Bottom: corresponding nuclei staining with Hoechst 33342 demonstrating a comparable amount of total cell number in all visual fields.

FIGURE S6. FPR1 is the dominant receptor in U87 cells. A, Ct values of all human FPRs from the RNA of U87 cells obtained through RT-qPCR, n=5, N=2. B, Ca^{2+} responses of U87 cells (green) or FPR-transfected HEK293T cells (red) towards 5 µM Aβ_{17-40} with (clear bars) or without (striped bars) co-application of 10 µM tBoc2. Signals were normalized to responses of the respective cells towards 10 µM WKYMVM, n=1, N=2 for HEK293T cells and N=3 for U87 cells. C, mean Ca^{2+} peak responses of U87 cells (green) or FPR-transfected HEK293T cells (red) that were treated with 10 nM SP4 (left) or 10 nM SP6 (right), n=1, N=3. All Error bars, S.D. One-way ANOVA test, Dunnett post hoc test; *, p ≤ 0.05, **, p ≤ 0.01, ***, p ≤ 0.001; ns, no significance

FIGURE S7. Validation of RT-qPCR primers for all human FPRs. Left: mean standard curves, regression line, and amplification efficiency (n = 3, N=3) generated by using a 10-fold serial dilution of a target DNA template starting with 0,1 ng of a purified and sequenced PCR product in t-RNA. Ct values were plotted against the log of template quantity for each dilution. Representative PCR products for all primer sets
were controlled by gel electrophoresis and sequencing for their specificity. Right: A representative data set of an amplification curve from these dilution series for each primer.

**FIGURE S8. Evidence for similar cell surface expression and calcium responses of FPR1 and FPR2 in HEK293T cells.** A, HEK293T cells were transfected with varying amounts of plasmid DNA for hFPR1 or hFPR2 and subsequently treated with 1 µM of FITC-labeled WKWVm-NH₂ to monitor their cell surface expression. Left: representative images of treated cells transfected with different DNA amounts Right: quantitative analysis of positively stained cells. Note that cell surface expression of both receptors upon dilution is reduced in a similar manner. *Error bars*, S.D. n=1, N=3 B, Mean Ca²⁺ peak responses of HEK293T cells transfected with decreasing amounts of human (red) or mouse (blue) FPR plasmid DNA. FPR1 and 2 of each species were treated with 1 µM of WKWVm-NH₂ and FPR3-transfected cells with 30 µM of WKWVm-CHO. Note that the amount of FPR 1 and 2 DNA can be diminished by more than 10-fold without a strong reduction of the signal amplitudes.

**SUPPORTING TABLE S1. Details of all used Aβ peptides.** The table details the manufacturers, catalog and lot numbers and purity of all Aβ peptides that were used within this work. Cal: Calcium Imaging, ChT: Chemotaxis, ThT: ThT Aggregation Assay. Buffer compositions are detailed in the experimental procedures.

**SUPPORTING TABLE S2. Details of all primers used for PCR experiments and Reverse Transcriptase.** Accession numbers and primer sequences which were used for RT-PCR, RT-qPCR and during Reverse Transcriptase.
Minimal requirements for the investigation of Aβ interactions with Formyl Peptide Receptors:

- Always validate Aβ effects with peptides from at least a second supplier.
- Include deviating and negative results with peptides from other sources.
- Always use single-use aliquots from a freshly dissolved frozen stock.
- Control stocks for ‘aging’ effects at least after six months.
- Monitor and report aggregation state and kinetics within your experiment time.
- Use low complexity buffer systems and avoid co-solvents if possible.
- Carefully describe all assay conditions such as peptide source, purity, solvent, assay buffer composition, pH and incubation time.