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 (FPRs) may contribute to inflammation in Alzheimer’s disease 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 are 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 Alzheimer’s disease through an interaction with different Aβ variants.

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 to 12 years after symptom onset (1). While there is an urgent need for therapies that may prevent or slow the progression of AD, no unequivocal treatment is currently available (2). 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 (3) and intracellular accumulation of hyperphosphorylated tau (4, 5) 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 3 decades old, the precise impact of Aβ on neuroinflammation and neurodegeneration is still incompletely understood (2). Major challenges in Aβ-research comprise the complex peptide processing involving multiple proteases (6), the challenging physiochemical properties of the resulting fragments that permit a formation of different oligomeric structures (7), and the pleiotropic physiological effects of Aβ-peptides on neurons, glia, and immune cells (8, 9).

Aβ is produced by a sequential cleavage of an amyloid precursor protein (APP) by β- and γ-secretase (10). Cleavage can occur at several sites, which results in the predominant production of peptides ending at position 38, 40, or 42 of the Aβ domain (10, 11). 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 (6, 12). 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 (7). There is clear evidence that the precise composition of these aggregates has a strong impact on their neurotoxicity (7, 13) and that different Aβ variants can trigger varying amounts of detrimental pro-inflammatory activities in astrocytes and microglia (14, 15). Their biochemical properties can vary significantly depending on the microenvironment in which they are generated, their amino acid composition, and their carboxyl terminus (13, 16). 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 central nervous system for pathological stimuli (14, 17). Upon activation, they undergo morphological changes and assume a reactive phenotype where they cease their supportive

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role (14). Instead, they obtain phagocytic and inflammatory functions, start to produce pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, and TNFs, and generate oxidative stress through the release of nitric oxide and reactive oxygen species (14, 18). Small oligomeric variants of Aβ are potent activators of microglia that lead to more severe neurotoxic outcomes than larger variants (19, 20). It is therefore crucial to understand how different Aβ variants interact with microglia to produce these neurotoxic effects. Unfortunately, interactions between Aβ 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) (21), scavenger receptors such as CD36, MARCO (Macrophage receptor with collagenous structure), and RAGE (receptor for advanced glycation endproducts) (22), and pattern recognition receptors (PRR) such as Toll-like receptors (23), NOD-like receptors, (24) and FPRs (formyl peptide receptors) (12).

The activation of PRRs by Aβ has become a recent focus in AD research because they have a special ability to potentially trigger the reactive state (14, 25). Among these PRRs, FPRs are one of the promising targets for AD research (12). They belong to a small gene family of G protein–coupled receptors with three members in humans and seven in mice (26). FPRs are primarily expressed in the innate and adaptive immune system, where they contribute to the detection and elimination of bacterial pathogens (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 (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 (36). Second, Aβ1-42 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 (35, 37). Third, FPR2-dependent recognition of Aβ1-42 led to the induction of oxidative stress, the release of pro-inflammatory cytokines, and chemotaxis of neutrophils and murine glial cells (36, 38–41). Fourth, in vitro expression studies demonstrated that Aβ1-42 can induce calcium mobilization and ERK signaling in cell lines that were transfected with human and murine FPR2 (35, 38, 42). In addition, FPRs mediate the intracellular uptake of Aβ1-42 in primary murine glia and in in vitro FPR expression systems (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 (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 (47). The only mouse gene that has a clear genetic ortholog inside the human gene family is mFpr1, which has a common ancestor with hFPR1 (48, 49). All others are paralogs, which evolved independently after the split of the human and mouse species (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 Aβ1-42, while data on the responses to other naturally occurring Aβ variants are lacking.

To address these questions, we used 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 identify N- and C-terminally abridged 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 Aβ1-42. Next, we can show that the solvent composition and manufacturer of the peptides critically influence the activation of FPRs by Aβ1-42. 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 Aβ1-40 and N-terminally abridged variants such as Aβ1-40, Aβ1-40, and Aβ1-40. Noteworthy, FPR1 is able to detect these peptides with up to 30-times higher sensitivity than Aβ1-42 and that these peptides induce calcium flux and chemotaxis in glial U87 cells that is likely mediated by hFPR1.

Results

 Activation of formyl peptide receptors by Aβ 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, mFrp-rs6, mFpr-rs7 were excluded from the analysis because they are not expressed in the brain (48) and likely do not mediate classical immune functions (50) but are responsible for the detection of yet largely unknown olfactory cues (50, 51). We first monitored changes in intracellular calcium levels of HEK293T cells transiently transfected with one of the different FPRs after application of Aβ1-42. Consistent with previous reports (36, 38), we observed that Aβ1-42 induced Ca2+ 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 Aβ1-42. 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 $A\beta_{1-42}$ than mFPR2, whereas the responses of human FPR1 were just slightly better than the ones of hFPR2. This raises the possibility that mFpr1 might be more relevant for the physiological responses to $A\beta_{1-42}$ in mouse models than its human ortholog. Second, we found an activation of human FPR3 by $A\beta_{1-42}$ 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 paralogs for $A\beta$ detection and signal transduction in between humans and mice, which should be further examined.

**Solvent and supplier variations can critically influence FPR responses to $A\beta$**

A detailed comparison of our results with other publications revealed some inconsistencies in the current literature. While our observation that $A\beta_{1-42}$ induces $Ca^{2+}$ flux through human and mouse FPR2 in micromolar concentrations is in accordance with all previous studies (38, 42), our study is the first to report of an activation of hFPR3. Next, our notion that mouse and human FPR1 are activated by $A\beta_{1-42}$ 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 et al. 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 dimethyl sulfoxide (DMSO) to dissolve $A\beta_{1-42}$, which can be critical in the case of FPRs because DMSO is an agonist for FPR1 and FPR2 and may therefore affect the $A\beta_{1-42}$-evoked signals (50). Second, varying types of physiological assay buffers and solvents were used, which might be critical in the case of $A\beta_{1-42}$ because this peptide tends to form diverse kinds of aggregates in different assay buffers (20, 52, 53). Third, $A\beta_{1-42}$ was obtained from different sources, which raises the possibility that...
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 min (clear bars) versus fluorescence after 120 min (striped bars). Buffer refers to ThT fluorescence without addition of peptides (gray 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 Ca²⁺ peak responses of human (red) or mouse (blue) FPRs to 10 μM 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 Ca²⁺ 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 Figure S1B. D, secondary structure composition of four Aβ1-42 peptides analyzed by circular dichroism (CD) spectroscopy; n = 3, N = 1; One-way ANOVA test, Tukey post hoc test. E, mean Ca²⁺ peak responses of cells transfected with human FPRs (red) or mock

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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 \( \text{A}\beta_{1-42} \) 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 Hexafluorosopropanol (HFIP) treated was used as positive control for aggregation because this peptide is known to readily form aggregates. Using a thioflavin T (ThT) assay (Fig. 2A), we first compared the aggregation status 10 min after dissolving the peptides with their kinetics over the course of the next 2 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 min after dissolving them in our assay buffer. In comparison to the peptide from P&E, \( \text{A}\beta_{1-42} \) 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 h revealed that the aggregation control with the HFIP–treated peptide showed the expected increase, whereas the status of \( \text{A}\beta_{1-42} \) 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 (TEM, Fig. S1A), \( \text{A}\beta_{1-42} \) 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, \( \text{Ca}^{2+} \) flux measurements also revealed clear differences in the response pattern of mouse and human FPRs to these peptides (Figs 2, B and C and S1B). \( \text{A}\beta_{1-42} \) from Sigma-Aldrich elicited a similar response pattern as that of P&E. However, it failed to activate hFPR1. When using \( \text{A}\beta_{1-42} \) 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 CD spectroscopy on \( \text{A}\beta_{1-42} \) peptides from P&E, Synpeptide, and the two variants from Anaspec, all dissolved in the assay buffer C1 (Figs. 2D and S1C). All tested peptides showed similar proportions of \( \alpha \)-helix and \( \beta \)-sheet but differed in the amount of \( \beta \)-turns and unstructured sequences. In comparison with the two different Anaspec peptides that elicited only relatively weak calcium responses, the \( \text{A}\beta_{1-42} \) peptides from P&E and Synpeptide, which were able to more robustly activate FPRs, displayed significantly elevated \( \beta \)-turn proportions. This suggests that increased proportions of \( \beta \)-turn conformation in \( \text{A}\beta \) 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 \( \text{A}\beta_{1-42} \) from P&E dissolved in our assay buffer C1 with those of the two frequently used buffers Hank’s balanced salt solution (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 (Fig. S2). Next, we carefully examined the effect of the commonly used solvent DMSO on the \( \text{A}\beta_{1-42} \) response pattern of the individual FPRs (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 \( \text{A}\beta_{1-42} \) 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 \( \text{A}\beta_{1-42} \) 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 \( \text{A}\beta_{1-42} \) but several other fragments such as \( \text{A}\beta_{1-40}, \text{A}\beta_{11-40} \), and \( \text{A}\beta_{17-40} \) 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 \( \text{A}\beta_{1-40} \) the N-abridged variants \( \text{A}\beta_{11-40} \) and \( \text{A}\beta_{17-40} \) and the C-terminally abridged fragments \( \text{A}\beta_{1-10} \) and \( \text{A}\beta_{1-16} \) (Fig. 3A). We observed that longer C-terminal deletions tend to be detrimental for the activation of FPRs because fragments such as \( \text{A}\beta_{1-10} \) and \( \text{A}\beta_{1-16} \) failed to induce any \( \text{Ca}^{2+} \) mobilization. In sharp contrast, the two tested N-abridged variants \( \text{A}\beta_{1-40} \) and \( \text{A}\beta_{11-40} \) elicited responses on all FPRs that we could previously activate with the full-length \( \text{A}\beta_{1-42} \). Unlike the responses to the full-length \( \text{A}\beta_{1-42} \), the

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(gray) towards 5 μM \( \text{A}\beta_{1-42} \) dissolved in the respective buffers; \( n = 3, N = 1 \), One-way ANOVA test, Dunnett post hoc test. F: comparison of ThT fluorescence of \( \text{A}\beta_{1-42} \) (P&E) dissolved in either C1, dimethyl sulfoxide (DMSO), Tris–NaCl, or HBSS. All assays were performed in the respective buffers. For experiments with peptides predissolved in DMSO, assays were conducted in C1 with a final concentration of DMSO: 0.2% (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. Aβ, amyloid beta; FPR, Formyl peptide receptor; ThT, thioflavin T.
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 ≤ 0.01; ***p ≤ 0.001; ns, no significance. Aβ, amyloid beta; FPR, Formyl peptide receptor.
FPR responses to these shorter fragments seemed to be less prone to manufacturer-dependent variations (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 10-fold higher sensitivity than the full-length peptide \( \text{A}_\beta_{1-42} \). (Fig. 3, B and C). Mouse and human FPR2 only responded to micromolar concentrations of these peptides (Fig. 3B). The observation that \( \text{A}_\beta_{11-40} \) and \( \text{A}_\beta_{17-40} \) can already induce an activation of mouse and human FPR1 at 30 to 100 nanomolar 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 \( \text{A}_\beta \) at micromolar concentration.

**N-abridged A\( \beta \) 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 \( \text{A}_\beta \), 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 \( \text{A}_\beta_{11-40} \) and \( \text{A}_\beta_{17-40} \) in U87 cells (Fig. 4) that are commonly used as a human glial cell model and are known for their natural expression of FPRs (56, 57). We first used reverse transcription polymerase chain reaction and reverse transcription quantitative

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**Figure 4.** N-abridged A\( \beta \) peptides induce hFPR1-dependent responses in glial U87 cells. A, mean Ca\(^{2+} \) peak responses of U87 cells after stimulation with different concentrations of \( \text{A}_\beta_{11-40} \) or \( \text{A}_\beta_{17-40} \). Striped bars indicate the response towards 10 \( \mu \)M of the positive control WKWVM-NH\(_2\), light gray and dark gray bars indicate negative controls; \( n = 3, N = 1 \). B, comparison of the Ca\(^{2+} \) responses upon stimulation with either 5 \( \mu \)M \( \text{A}_\beta_{11-40} \) or \( \text{A}_\beta_{17-40} \) alone (green bars) or in the presence of 10 \( \mu \)M of the competitive FPR-antagonist tBoc2 (black bars); \( n = 3, N = 1 \). C, dose-dependent chemotaxis of U87 cells upon stimulation with either \( \text{A}_\beta_{11-40} \) or \( \text{A}_\beta_{17-40} \). 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 \( \mu \)M fMLF, light gray bars show migration without stimuli, and dark gray 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 \( \mu \)M tBoc2 (black bars) towards 1 \( \mu \)M of \( \text{A}_\beta_{11-40} \) or \( \text{A}_\beta_{17-40} \); \( n = 3, N = 1 \). All Error bars, S.D. One-way ANOVA test, Dunnett post hoc test for A and C and t test for B and D; \( ^* p \leq 0.05; ^{**} p \leq 0.01; ^{***} p \leq 0.001; \text{ns, no significance. A}_\beta, \text{amyloid beta; FPR, Formyl peptide receptor.} \)
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denose chain reaction to examine the expression of the individual FPRs in these cells. Our data revealed that these cells show high mRNA levels of hFPRI but only a very modest expression of hFPRII and hFPRIII (Figs. 5A and S6A and S7). Immunocytochemistry with receptor subtype-specific antibodies (Figs. 5B and S5) confirmed a robust expression of hFPRI and lower amounts of hFPRII. Interestingly, hFPRIII 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. 4, A and B) that were closely similar to the typical FPR time kinetics (50) observed in the transfected HEK cells (Fig. 1). In chemotaxis experiments, only Aβ11-40 but not Aβ17-40 induced migration of these cells (Fig. 4, C and D). Both, the calcium signals and the chemotaxis, were FPR dependent because a co-application of tBoc2, which is a competitive antagonist of FPRII and FPRIII but does not inhibit FPRI (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 hFPRI (Fig. 5C). Next, we used receptor subtype-specific activators to test which receptor can trigger a calcium signal. To this end, we challenged U87 cells and hFPRI-transfected HEK293T cells with two bacterial signal peptides SP6 and SP4 at concentrations where they would only activate either hFPRI or hFPRIII, respectively (50). Of note, both compounds would not activate hFPRIII (Fig. S6C). In line with our previous notion that responses to the N-abridged Aβ variants likely depend on hFPRI, the U87 cells only responded to the hFPRI-specific SP6 stimulus with a calcium signal but not to the hFPRII-specific SP4 stimulus (Figs. 5D and S6C). Next, we performed cross-desensitization experiments, which showed that responses of U87 cells towards Aβ17-40 were absent after pre-application of the FPRII-specific agonist SP6 but were not influenced by pre-application of the FPRII-specific agonist SP4 (Fig. S5E). Finally, in the chemotaxis assay, U87 cells only migrated towards the FPRI-specific SP6 but not towards the FPRII-specific SP4 (Fig. 5F). Taken together, these experiments strongly argue that the sensitive calcium and chemotaxis responses of U87 to N-abridged Aβ variants depend on an activation of hFPRI.

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 hFPRIII by Aβ and the first to identify N-abridged Aβ fragments as activators of FPRs. Moreover, our data show that hFPRI and its mouse ortholog 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 FPRII might be more relevant for the pro-inflammatory physiological responses to Aβ than FPRI. Interestingly, several independent lines of evidence from literature are highly consistent with this hypothesis. First, FPRI is already expressed at the resting state of glial cells and is further upregulated during their transformation into the reactive state (59). Next, mFpr1 is highly upregulated in the cortex and hippocampus of transgenic APP/PS1 mice, which are a commonly used AD animal model (35). Moreover, FPRIII 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 (14, 35), whereas several reports suggest an anti-inflammatory role of FPRII (41, 44, 60). Next, treatment of APP/PS1 mice with the competitive FPR antagonist tBoc2 that preferentially binds to FPRIII (26) leads to reduced microglia reactivity, decreased neuronal pathology, and improved cognitive performance (46). Taken together, these findings strongly support our notion that FPRII and other FPR variants contribute 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 (44, 61), Lipoxin A 4 (62, 63), Resolvin (63), or mitochondrial peptides (64) may further modulate these signals depending on their local concentration. However, the observed 10- to 30-fold higher affinity of FPRII 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 cerebrospinal fluid at similar concentrations as Aβ1-42 and is furthermore an integral part of senile plaques (54, 65, 66). Aβ17-40/42 is thought to occur at 4-fold higher rates than Aβ1-42 and Aβ11-40/42, however, its exact quantification is difficult and therefore often disregarded (55). 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 (53, 67). 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 (53, 68). Thus, to the best of our knowledge, our data are consistent with a model in which recognition of these N-abridged Aβ variants via FPRI could thus occur under physiological 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

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Figure 5. The activation of U87 cells by 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 reverse transcription polymerase chain reaction with primers for all hFPRs. Right: Quantification of complementary DNA for all FPRs obtained through reverse transcription quantitative polymerase chain reaction, n = 5, N = 2. Details on the primer efficiency, specificity, and linearity are given in Figures S6 and S7. One-way ANOVA test, Dunnett post hoc test. 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 for exposure time, brightness and contrast pictures are shown in Figure S5. Scale bars indicate 100 μm. Right: Quantification of the mean FPR staining in U87 cells (green) in comparison to FPR-transfected HEK293T cells (red). Analysis was performed on images acquired with the same settings. n = 2,
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 (20, 52, 53). For example, Szczepanik et al. (53) 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 fibers captured by TEM images and variations in the secondary structure as indicated by our CD. In accordance with this idea, in previous studies, both monocytes and microglia lost FPR-dependent signaling with progressing aggregation of Aβ1-42 (42, 69). Of note, it is conceivable that chemical modifications of the peptide, for example, 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 (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 second supplier. Next, we would like to encourage researchers 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 cosolvents such as DMSO if possible. In case DMSO or other cosolvents 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 cosolvents in an assay should be reported because this all can critically influence the results.

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N = 5 for U87 cells and n = 2 for HEK293T cells. Evidence for closely similar staining intensities of FPR1 and FPR2 by the used protocols is shown in Figure S5. One-way ANOVA test, Tukey post hoc test. C, the concentration-dependent Ca2+ responses of HEK293 cells and U87 cells towards the N-abridged fragments Aβ1-40 and Aβ17-40 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 Aβ1-40 are abolished by the FPR1 activator SP6 but not by the FPR2 agonist SP4. Representative Ca2+ 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. Aβ, amyloid beta; FPR, Formyl peptide receptor.

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

Figure 6. Minimal requirements for the investigation of Aβ interactions with FPRs. Aβ, amyloid beta; FPR, Formyl peptide receptor.

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 subcloned into pcDNA3.1(+)(Invitrogen) as previously described (50).

Ligands and chemicals

Purity of all peptides was at least >95%. Details on manufacturer, Catalog numbers, lot numbers, exact purity of all peptides, solvents, and storage conditions are given in Table S1. The amino acid composition of all Aβ peptides corresponded to the human WT sequence. Aβ1-42 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 to 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 (Table S1) where we investigated the effects of different solvents Aβ1-42 from AnaSpec/MobiTec, P&īE, and Synpeptide were dissolved at 5 mM in DMSO (Sigma-Aldrich), 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. Aβ₁₋₁₀, Aβ₁₋₁₆, and Aβ₁₋₁₆ were purchased from Anaspec/MoBiTec. The Aβ₁₋₇₋₄₀ peptide used throughout the main text was purchased from Anaspec/MoBiTec and validated with Aβ₁₋₇₋₄₀ peptides from Sigma-Aldrich and Synepeptide. Aβ₁₋₁₄₋₄₀ 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. To their high hydrophobicity, Aβ₁₋₁₀ and Aβ₁₋₇₋₄₀ were dissolved in DMSO at 5 mM. Tert-Boc-FLFL (tBoc2) was purchased from Bachem and dissolved in DMSO at 30 mM. f-MLFFYS (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 2 mM L-glutamine (Biowest) until 80% confluence. For transfection, approximately 2000 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 24 h 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 Gα₁₆ since this subunit is needed to trigger FPR-dependent calcium signals in HEK293 cells (50, 70). The medium of transfected cells was changed 24 h 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 (Biowest) supplemented with 10% (v/v) heat-inactivated FCS (Pan Biotech), 1 unit/ml penicillin-streptomycin (Biowest), 2 mM L-glutamine (Biowest), and 1 unit/ml non-essential amino acids (Biowest) until 50 to 60% confluence. For calcium imaging and immunostaining, on average 2500 cells were seeded in each well of PDL-coated (10 μg/ml in PBS, Sigma) black optical 96-well μCLEAR-plates (Greiner Bio-One).

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**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 Calibryte 520 AM (AAC Bioquest) for 2 h 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 48 h after transfection, while experiments with U87 cells were performed 24 h 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 by the appropriate buffer and solvent controls and with 10 μM WKWMVm-NH₂ 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 (Biowest) supplemented with 0.5% (v/v) heat-inactivated FCS (Pan Biotech), 1 unit/ml penicillin-streptomycin (Biowest), 2 mM L-glutamine (Biowest), and 1 unit/ml non-essential amino acids (Biowest). Approximately, 1000 cells were placed into the top chamber of each well of a 96-well ClearView Plate (EssenBioscience). Chemotacticantants 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, 2000 HEK293T cells or 3500 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. Twenty four hours after transfection, cells were fixed with 4% (v/v) methanol-free paraformaldehyde (Polyscience Inc) in PBS for 30 min at RT and afterward 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.
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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 reverse transcription polymerase chain reaction experiments were performed using DreamTaq DNA polymerase (ThermoFisher Scientific) with primers (Sigma-Aldrich) comprising the full coding region with complementary DNA (cDNA) obtained from 0.3 ng total RNA in a total reaction volume of 20 μl. PCR conditions were as follows: 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 60 s, followed by a final extension of 72 °C for 10 min. qPCRs were performed with the Biozym Blue SPCR Kit according to the MIQE guidelines (71). Reverse transcription quantitative polymerase chain reaction reactions were carried out with cDNA obtained from 0.15 ng total RNA as duplicates. qPCRs for calibration curves, a dilution series of a sequenced c standard curves supplemented in Figure S6.

CD spectroscopy
Thirty micromolar of Aβ1-42 dissolved in C1 were sonicated for 10 min 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
Thirty micromolar 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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Approximately, 2000 HEK293T cells were seeded in each well of a PDL-coated black optical 96-well plate and transfected as described above. Forty eight hours after transfection, cells were treated with 1 μM of FITC-labeled WKWVm-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).

ThT aggregation assay
For the experiments in C1 buffer, Aβ1-42 peptides were dissolved as 30 μM stock solutions For experiments with different buffers, Aβ1-42 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 Aβ1-42 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, 2000 HEK293T cells were seeded in each well of a PDL-coated black optical 96-well plate and transfected as described above. Forty eight hours after transfection,
DGF Project number 316249678 - SFB 1279 (C04) to Q. L., DGF Project number 441734479 to C. V. S., and INST 252/19-1 FUGG to B. B.

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

Abbreviations—The abbreviations used are: AD, Alzheimer’s disease; APP, amyloid precursor protein; Aβ, amyloid beta; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FPR, Formyl peptide receptor; HFIP, Hexafluorosopropanol; PDL, poly-D-lysine; PRR, pattern recognition receptor; TEM, transmission electron microscopy; ThT, thioflavin T.

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