Effects of Transmitters and Amyloid-Beta Peptide on Calcium Signals in Rat Cortical Astrocytes: Fura-2AM Measurements and Stochastic Model Simulations

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Abstract

**Background:** To better understand the complex molecular level interactions seen in the pathogenesis of Alzheimer’s disease, the results of the wet-lab and clinical studies can be complemented by mathematical models. Astrocytes are known to become reactive in Alzheimer’s disease and their ionic equilibrium can be disturbed by interaction of the released and accumulated transmitters, such as serotonin, and peptides, including amyloid-β peptides (Aβ). We have here studied the effects of small amounts of Aβ25–35 fragments on the transmitter-induced calcium signals in astrocytes by Fura-2AM fluorescence measurements and running simulations of the detected calcium signals.

**Methodology/Principal Findings:** Intracellular calcium signals were measured in cultured rat cortical astrocytes following additions of serotonin and glutamate, or either of these transmitters together with Aβ25–35. Aβ25–35 increased the number of astrocytes responding to glutamate and exceedingly increased the magnitude of the serotonin-induced calcium signals. In addition to Aβ25–35-induced effects, the contribution of intracellular calcium stores to calcium signaling was tested. When using higher stimulus frequency, the subsequent calcium peaks after the initial peak were of lower amplitude. This may indicate inadequate filling of the intracellular calcium stores between the stimuli. In order to reproduce the experimental findings, a stochastic computational model was introduced. The model takes into account the major mechanisms known to be involved in calcium signaling in astrocytes. Model simulations confirm the principal experimental findings and show the variability typical for experimental measurements.

**Conclusions/Significance:** Nanomolar Aβ25–35 alone does not cause persistent change in the basal level of calcium in astrocytes. However, even small amounts of Aβ25–35, together with transmitters, can have substantial synergistic effects on intracellular calcium signals. Computational modeling further helps in understanding the mechanisms associated with intracellular calcium oscillations. Modeling the mechanisms is important, as astrocytes have an essential role in regulating the neuronal microenvironment of the central nervous system.

Introduction

Alzheimer’s disease (AD) is a progressive and irreversible neurodegenerative disorder that leads to cognitive impairment and emotional disturbances. Symptoms result from the degeneration of brain tissue, seen as shrinkage of certain brain regions, which are involved in cognitive processes, learning, and memory formation (reviewed in [1]). In addition to brain shrinkage, AD patients suffer from accumulation of amyloid-beta (Aβ) containing neuritic plaques and neurofibrillary tangles (tau protein in neuronal somata), which are considered as hallmarks of AD. Though the pathological changes in the brain can be detected using MRI and PET imaging techniques, the exact molecular mechanisms leading to the severe symptoms are not yet known. Early diagnosis together with a possibility of specific targeted treatment would provide the patients with more years of quality life.

Amyloid plaques containing aggregated Aβ fragments have been shown to disturb the homeostasis of intracellular calcium ions (Ca²⁺) and contribute to the altered Ca²⁺ signaling in the brain cells [1]. The plaques typically consist of 39–42 amino acid Aβ fragments, and the plasma ratio of 42 and 40 amino acids long fragments (Aβ42/Aβ40) is suggested of being useful for identifying the risk of developing mild cognitive impairment and AD [2,3]. Based on the classification of amino acids by Branden and Tooze [4], 25 amino acids out of the total 42 have hydrophobic side chains in Aβ42. Therefore, Aβ42 tends to aggregate easier than the shorter Aβ fragments. Aβ42 and the shorter 11 amino acids...
long synthetic derivative (Aβ25–35) are both fragments which are widely used in Alzheimer's disease research [see recent studies [5–10]] with specifically Aβ25–35 having Ca²⁺-mediated neurotoxic properties [11,12].

So far the *in vitro* studies of the effects of Aβ peptide on the cellular Ca²⁺ responses have failed to give any definite answers to the mechanisms involved. Together with the longer fragments, Aβ25–35 has been shown to depress hippocampal long-term potentiation [13] and to potentiate the long-term depression [14], both of which depend on the increases in intracellular Ca²⁺ concentration in neurons. Aβ25–35 has been shown to induce transient changes in intracellular Ca²⁺ concentration in astrocytes [15,16]. These effects may be important in explaining the loss of new memory formation and learning seen in AD. The detailed mechanisms behind the Aβ-induced neuronal and glial Ca²⁺ fluctuations, as well as the changes triggered by these, require further studies.

One of the central functions of astrocytes is neurotransmitter/neurotransmitter release and uptake in the neuronal synaptic cleft of the tripartite synapse [17] together with more complex regulation of the neuronal microenvironment [18–23]. Astrocytes thus have a vital role in the synaptic information processing and in the metabolism of the central nervous system. Astrocytes release transmitters and have receptors and transporters for different neurotransmitters in their plasma membranes, such as for serotonin (5-hydroxytryptamine; 5-HT), ATP, and glutamate [17,24]. Astrocytes, as well as other glial cells, use both spontaneous and stimulated variations of the Ca²⁺ concentration for intra- and intercellular signaling [25,26]. Previous electrophysiological and Ca²⁺ imaging studies have shown how already micromolar concentration of 5-HT cause transient release of Ca²⁺ from intracellular stores followed by prolonged transmembrane inward Ca²⁺ flow [17,27]. We here have used rat cortical astrocytes, similarly to our earlier studies on Aβ25–35 and Aβ1–40 [16], to study the special effects of Aβ25–35 to Ca²⁺ signals when added together with transmitters. We now show that Aβ25–35 increases the initial peak of Ca²⁺ release when added together with 5-HT, compared to the effects of 5-HT alone.

Despite the rapid advancements in computing technology, it is currently not possible to model mathematically the biological systems of realistic complexity over interesting time scales by only using the molecular dynamic approach [28]. Typically, the details of the state of the system (such as the position, orientation, and momentum of individual particles) are excluded in the modeling of whole-cell level phenomena. Here, we describe a model of astrocyte Ca²⁺ signals as a macroscopic flow of Ca²⁺ ions rather than as a model of each individual Ca²⁺ channel in the membranes. In the case of AD, abnormal Ca²⁺ signals could be among the first hallmarks of disturbed brain function (the correlation between Ca²⁺ and Aβ is reviewed in [29]). A computational model which closely mimics the experimentally measured Ca²⁺ signals in rat cortical astrocytes helps in understanding the interaction of the various components of Ca²⁺ dynamics in healthy cells versus the cells with dysfunctional metabolism.

**Methods**

**Experimental methods and data**

**Ethics Statement.** Confluent primary astrocyte cultures were prepared from cortices of newborn Sprague-Dawley rat pups as previously described [30], with minor modifications. Pups were killed by decapitation according to the procedure conforming to the Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Albany Medical College Institutional Animal Care and Use Committee for Dr. H.K. Kimelberg, Protocol ID 006038 entitled “Neurotransmitter receptors and ion channels on astrocytes”.

**Cell culture.** Primary astrocyte cultures were prepared from new-born Sprague-Dawley rat pups. In brief, the cerebral hemispheres were removed, freed from the meninges and mechanically dissociated using Digestase (Sigma, St. Louis, MO, USA) into culture medium (Eagle’s Medium with Earle’s salts, Gibco, U.K.) supplemented with 10% heat-inactivated horse serum (HS, Gibco, U.K.), 25 mM sodium bicarbonate and antibiotics (penicillin and streptomycin). Deoxyribonuclease I (Sigma, St. Louis, MO, USA) was added to prevent cell clumping during the second extraction. Cells were grown on coverslips in culture dishes and kept at 37°C in an air-ventilated humidified incubator containing 5% CO₂. The medium was first changed after one day and subsequently twice a week. About 95% of the cells routinely stained positively for glial fibrillary acidic protein (GFAP⁺), with polyclonal rabbit anti-cow GFAP used as the primary antibody and either rhodamine or fluorescein conjugated gamma & light chain goat anti-rabbit IgGs as secondary antibody. The studies were performed on cells kept for 1 to 4 weeks in culture.

**Calcium imaging.** Fura-2-acetoxymethyl ester (Fura-2-AM) is a membrane penetrating derivative of the radiometric Ca²⁺ indicator Fura-2 used to measure intracellular Ca²⁺ concentrations by fluorescence. Inside the cell, the acetoxymethyl groups in Fura-2-AM are removed by cellular esterases resulting to Fura-2, the pentacarboxylate Ca²⁺-imaging Ringer (mM): NaCl 122, KCl 3.3, MgSO₄ 0.4, CaCl₂ 1.3, KH₂PO₄ 1.2, HEPES 25, Glucose 10, and sucrose to balance the osmolarity to ~ 320 mOsmol, pH 7.35–7.4. The medium was first changed after one day and subsequently twice a week.

**Procedure.** The solutions. 1. Ca²⁺-imaging Ringer (mM): NaCl 122, KCl 3.3, MgSO₄ 0.4, CaCl₂ 1.3, KH₂PO₄ 1.2, HEPES 25, Glucose 10, and sucrose to balance the osmolarity to ~ 320 mOsmol, pH 7.35–7.4. 2. Aβ25–35 peptide (RBI, Natick, MA, USA) was first dissolved in water and then diluted in buffer solution. 10 nM, 200 nM and 1 μM final concentration of the peptide was used either acutely or with incubation. 3. 1 or 10 μM final concentration of 5-HT HCl (for references see [17,27]), or 50 μM L-glutamate were added in the recording chamber.
The obtained experimental data together with the known components affecting cellular Ca\(^{2+}\) concentration (presented in Figure 1) were used to design the type of the computational model. Because of the variability of detected Ca\(^{2+}\) levels (see Figure 2), stochasticity was introduced into the computational model which was validated by the data obtained from Fura-2AM measurements.

### Stochastic model for Ca\(^{2+}\) signals

Computational modeling, in general, means mathematical description of the functional properties of the system components and the analysis of the model predictions. One of the challenges in computational modeling is the lack of precise experimental data for model components. In other words, a specific experimental data set with proper statistics is needed for selecting relevant range of values for model parameters. Validation of the model is typically done by comparing the predicted output of the model with the experimental data. To ensure the relevant parameter values, a computational model for Ca\(^{2+}\) signaling in astrocytes by Di Garbo et al. [31] was taken as a reference model. The model takes into account the physiological phenomena known to be the major contributors in the intracellular Ca\(^{2+}\) oscillations. In summary, it describes the Ca\(^{2+}\) concentration in cytosol as a six-component system (a graphical illustration in Figure 1). Namely, 1) Ca\(^{2+}\) leak from/to extracellular matrix (ECM), 2) capacitive Ca\(^{2+}\) entry (CCE) from ECM, 3) Ca\(^{2+}\) entry via ionotropic receptors, 4) Ca\(^{2+}\) leak from intracellular stores, such as endoplasmic reticulum (ER), 5) storage of Ca\(^{2+}\) to ER via sarco(endo)plasmic Ca\(^{2+}\) ATPase (SERCA) pumps, and 6) Ca\(^{2+}\) release from ER mediated by inositol 1,4,5-trisphosphate (IP\(_3\)). The reference model carefully addresses a widely accepted mechanism for astrocytic Ca\(^{2+}\) increases via the canonical G protein/phospholipase C (PLC)/IP\(_3\) pathway [32] where the IP\(_3\) released into the cytosol binds to its receptor (IP\(_3\)R) on ER, the Ca\(^{2+}\) channels open, and Ca\(^{2+}\) ions inside the ER are liberated to cytosol causing a sharp rise in the cytosolic concentration of free Ca\(^{2+}\) which is normally kept very low (see also [33,34]). The parameters used for both the reference model (deterministic; Di Garbo et al. [31]) and the here developed stochastic model are presented in Table 1.

The kinetics of biological processes are typically stochastic, i.e. random, in nature [28]. Therefore, the cellular functions cannot be properly understood with purely deterministic models (see, e.g., [35–37]) and both the intrinsic and extrinsic stochastic phenomena need to be accounted for in silico models. Intrinsic stochasticity is caused by the dynamics of the system from the random timing of individual reaction events. The importance of intrinsic stochasticity becomes obvious in systems with low numbers of molecules. However, stochasticity included in the model may not always be able to explain the large diversity observed in experimental measurements (as shown in [38]). The low numbers make individual reaction events, which change molecular numbers by one or two, more significant. At the same time the extrinsic stochasticity is caused by the system interacting with other stochastic systems in the cell or its environment. Mathematically, stochasticity means that the trajectories for each simulation are slightly different from one another and computationally intensive simulations are often required to follow the time evolution of the system dynamics. Our earlier studies [39,40] have shown the potential of stochastic differential equations in the kinetics of signal transduction and ion channels. Mathematical analysis alone may be able to completely describe all the properties of interest in the case of simple random systems. However, mathematical analysis is...
not possible for more complex stochastic models, i.e. the complex
stochastic models are analytically intractable.

The exact method to model chemical reactions, when diffusion is not taken into account, is the discrete-state chemical master equation (CME, [41]). However, the CME can rarely be solved and thus an algorithm called Gillespie stochastic simulation algorithm (SSA, [42,43]) has been developed. The SSA presents an easy way to simulate the actual CME process and it is used more and more in computational modeling studies. In many cases, the SSA is slow to simulate and thus, we have chosen to introduce stochasticity into the reference model [31,44] by the chemical Langevin equation (CLE, [45]), that is one type of stochastic differential equation. The CLE represents the continuous-state Markov model approximated from the exact CME. The CLE is much faster to simulate than the actual SSA when large volumes are considered but it can produce negative values when low concentrations are simulated [39]. However, for the system modeled in this study the CLE produce realistic results and can be thus used. When making the stochastic extension of the model, we need to assume volumes for the cytosol (Vcytosol) and ER (VER) (see Table 1 for more information).

To describe the time-series behavior of the model, a set of equations (Equations 1–4) was introduced. \([Ca^{2+}]_{cyt}, [Ca^{2+}]_{ER}\) and \([IP_3]\) represent concentrations of cytosolic \(Ca^{2+}\), \(Ca^{2+}\) in the ER, and cytosolic \(IP_3\) respectively. The fraction of active \(IP_3\) receptors on the ER membrane was termed \(IP_3^R\). In the stochastic terms of Equations 1–4, \(\vec{W} = \{W_1, ..., W_12\}^T\) stands for the Brownian motion and \(\vec{W}(t) \sim N(0, I)\). Furthermore, \(N_A\) represents the Avogadro’s number.

\[
d[Ca^{2+}]_{cyt} = (v_{LM} + v_{CCE} + v_{rel} - v_{OUT} + v_{ER}\text{leak} + v_{ER}\text{rel}) dt + \frac{1}{\sqrt{N_A V_{cytosol}}} \sqrt{v_{LM} dW_1} + \sqrt{v_{CCE} dW_2} + \sqrt{v_{rel} dW_3} - \sqrt{v_{OUT} dW_4} + \sqrt{v_{ER}\text{leak} dW_5} + \sqrt{v_{ER}\text{rel} dW_6} - \sqrt{v_{SERCA} dW_7} - \sqrt{v_{ER}\text{leak} dW_8} - \sqrt{v_{ER}\text{rel} dW_9}
\]

\[
d[Ca^{2+}]_{ER} = \beta(v_{SERCA} - v_{ER}\text{leak} - v_{ER}\text{rel}) dt + \frac{1}{\sqrt{N_A V_{ER}}} (\sqrt{v_{SERCA} dW_{10}} - \sqrt{v_{ER}\text{leak} dW_{11}} - \sqrt{v_{ER}\text{rel} dW_{12}})
\]

\[
d[IP_3]^R = (v_{IP_3\text{Rec}} - v_{IP_3\text{Inc}}} dt + \frac{1}{\sqrt{N_A V_{cytosol}}} (\sqrt{v_{IP_3\text{Rec}} dW_8} - \sqrt{v_{IP_3\text{Inc}}} dW_9)
\]

\[
d[IP_3] = (v_{PLC}\beta + v_{PLC} - v_{IP_3}(\text{Deg})\) dt + \frac{1}{\sqrt{N_A V_{cytosol}}} (\sqrt{v_{PLC}\beta dW_{10}} + \sqrt{v_{PLC}\text{Deg} dW_{11}} - \sqrt{v_{IP_3}(\text{Deg}) dW_{12}})
\]

Due to the lack of fully understanding the phenomena related to CCE, the rate regulating capacitive \(Ca^{2+}\) influx was assumed to be a nonlinear function of \([Ca^{2+}]_{ER}\), as described in [31]:

\[
v_{CCE} = \frac{k_{CCE} H_{CCE}^2 + [Ca^{2+}]^2_{ER}}{H_{CCE}^2 + [Ca^{2+}]^2_{ER}}.
\]

Earlier experimental results (cited in [31]) indicated that the transient component in cytosolic \(Ca^{2+}\) concentration was induced by the activation of the metabolotropic receptor (mR) due to stimuli/input-evoked \(Ca^{2+}\) release from the intracellular stores, whereas the activation of the ionotropic receptor (iR) mediated the sustained component (similarly to our results; see Results and Figure 3A). In the reference model by Di Garbo et al. [31], ATP has an effect on \([Ca^{2+}]_{cyt}\) via both ionotropic and metabotropic receptors. The same is here assumed to 5-HT. The parts of

| Table 1. Model parameters and used parameter values of the computational model. |
|----------------------------------|-----------------|---------------------------------|
| Symbol | Value | Explanation |
| \(k_0\) | 0.03 \(\mu M/s\) | Rate of \(Ca^{2+}\) leak across the plasma membrane |
| \(k_1\) | 0.0004 \(1/s\) | Rate of \(Ca^{2+}\) leak from the ER |
| \(k_2\) | 0.2 \(1/s\) | Rate of \(Ca^{2+}\) release through \(IP_3\) receptor |
| \(k_3\) | 0.5 \(1/s\) | Rate constant of SERCA pump |
| \(k_4\) | 0.5 \(1/s\) | Rate of \(Ca^{2+}\) extrusion from plasma membrane |
| \(k_6\) | 4 \(1/s\) | Rate constant of \(IP_3\) receptor inactivation |
| \(k_7\) | 0.08 \(1/s\) | Rate constant of \(IP_3\) degradation |
| \(v_{c}\) | 0.02 \(\mu M/s\) | Rate constant of PLC\(\beta\) |
| \(K_{IP_3}\) | 0.3 \(\mu M\) | Half saturation constant for \(IP_3\) activation of the corresponding receptor |
| \(K_a\) | 0.2 \(\mu M\) | Half saturation constant for \(Ca^{2+}\) activation of the \(IP_3\) receptor |
| \(K_i\) | 0.2 \(\mu M\) | Half saturation constant for \(Ca^{2+}\) inhibition of the \(IP_3\) receptor |
| \(K_C\) | 0.3 \(\mu M\) | Half saturation constant for \(Ca^{2+}\) activation of PLC\(\beta\) |
| \(\beta\) | 35 | Ratio of the effective volumes for \(Ca^{2+}\) of cytoplasm and ER |
| \(H_{CCE}\) | 10 \(\mu M\) | Half inactivation constant for CCE influx |
| \(k_{CCE}\) | 0.01 \(\mu M/s\) | Maximal rate constant for CCE influx |
| \(k_{R}\) | 0.08 \(\mu M/s\) | Maximal rate of stimuli-evoked ionotropic \(Ca^{2+}\) influx |
| \(H_{R}\) | 0.9 \(\mu M\) | Half saturation constant for stimuli-evoked ionotropic \(Ca^{2+}\) influx amplitude |
| \(k_{rel}\) | 0.5 \(\mu M/s\) | Maximal rate of \(IP_3\) production mediated by the metabotropic receptor |
| \(\beta_D\) | 10 \(\mu M\) | Dissociation constant for the binding of ligand/ metabolotropic receptor |
| \(V_{cell}\) | 24 \(\beta\) | Volume of the cell |
| \(V_{ER}\) | 0.2 \(\times V_{cell}\) | Volume of the ER |
| \(V_{cytosol}\) | 0.4 \(\times V_{cell}\) | Volume of the cytosol |

Model parameters and parameter values used both in the deterministic reference model and in the stochastic model introduced in this study, excluding the last three volumes which were only used in the stochastic model. More information and references for the used values can also be found from [31]. Used abbreviations: capacitive \(Ca^{2+}\) entry (CCE), endoplasmic reticulum (ER), inositol 1,4,5-trisphosphate (IP\(_3\)), sarco(endoplasmic \(Ca^{2+}\) ATPase (SERCA), and phospholipase \(C\) (PLC). doi:10.1371/journal.pone.0017914.t001

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Figure 3. Changes in cytosolic Ca\textsuperscript{2+} concentration induced by 5-HT (A–B) and computational simulations (C–D). A. Fast Ca\textsuperscript{2+} transient and a more sustained component are seen when 10 \textmu M 5-HT is added at t = 80 s. B. Changes in cytosolic Ca\textsuperscript{2+} concentration induced by 1 \textmu M 5-HT at t = 170 s in Ca\textsuperscript{2+} free media. Fast Ca\textsuperscript{2+} transient is seen but the more sustained component is cut off. C. Model simulations of changes in cytosolic Ca\textsuperscript{2+} concentration induced by 10 \textmu M input at t = 80 s. Fast Ca\textsuperscript{2+} transient and a more sustained component are seen. D. Model simulations of changes in cytosolic Ca\textsuperscript{2+} concentration induced by 1 \textmu M input at t = 170 in simulated Ca\textsuperscript{2+} free media conditions: model rates \(v_{CM},v_{CEM}\), and \(v_R\) are set to zero. Fast Ca\textsuperscript{2+} transient is seen but the more sustained component is cut off.

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the model (Equations 6 and 7) describing the ATP-induced Ca\textsuperscript{2+} response in [31] is here used with some modifications to activate the model for astrocytic Ca\textsuperscript{2+} signaling with 5-HT and \(\Delta \beta\).

In this study, the rate of Ca\textsuperscript{2+} influx, induced by ionotropic receptors, from ECM to cytosol was modeled as in [31]:

\[ v_R = k_R \frac{[\text{input}]}{[\text{input}]}^{1.4} \]  \hspace{1cm} (6)

Similarly, the activation of G protein and PLC\(\beta\) pathways, induced by metabotropic receptors, to promote the IP\(_3\) production were reformulated from [31,46] and modeled as:

\[ v_{PLC\beta} = k_{mR} \frac{[\text{input}]}{[\text{input}]} \]  \hspace{1cm} (7)

The remaining rate terms used in Equations 1–4 were taken from [46] and are explicitly formulated as Equations 8–16.

\[ v_{LM} = k_0 \]  \hspace{1cm} (8)

\[ v_{OUT} = k_5[Ca^{2+}]_{cyt} \]  \hspace{1cm} (9)

\[ v_{ER\text{(leak)}} = k_1([Ca^{2+}]_{ER} - [Ca^{2+}]_{cyt}) \]  \hspace{1cm} (10)

\[ v_{ER\text{(calc)}} = \frac{k_2 IP_3 [Ca^{2+}]_{cyt}^2 [IP_3]^2}{(K_2 + [Ca^{2+}]_{cyt}^2/K_{IP_3} + [IP_3]^2)}([Ca^{2+}]_{ER} - [Ca^{2+}]_{cyt}) \] \hspace{1cm} (11)

\[ v_{SERCA} = k_3[Ca^{2+}]_{cyt} \]  \hspace{1cm} (12)

\[ v_{IP_3\text{(Dec)}} = k_9[IP_3] \]  \hspace{1cm} (13)

\[ v_{PLC\beta} = \frac{v([Ca^{2+}]_{cyt}^2)}{K_2 + [Ca^{2+}]_{cyt}^2} \]  \hspace{1cm} (14)

\[ v_{IP_3\text{(Inact)}} = k_4 K_2 \]  \hspace{1cm} (15)

In addition, the following initial values were used: \([Ca^{2+}]_{cyt} = 6 \cdot 10^{-9} M\), \([Ca^{2+}]_{ER} = 7.2 \cdot 10^{-5} M\), \([IP_3] = 9.6 \cdot 10^{-9} M\), and \(IP_3 R = 0.9174\).

Results

To specifically study how the non-aggregated A\(\beta\)25–35 affects the metabotropic 5-HT receptor function, we added small amyloid peptide concentrations together with the transmitter and measured the ratio of emissions at 340 and 380 nm in Fura-2AM loaded rat cortical astrocytes in primary cultures. In some experiments L-glutamate was also added in aim to study the possible differences between glutamate and 5-HT receptor activation in these cells. The ratio of emissions is directly correlated to cytosolic Ca\textsuperscript{2+} concentration \([Ca^{2+}]_{cyt}\). A deterministic model, introduced by Di Garbo et al. [31], was used as a reference model to which stochasticity was introduced by CLE in aim to reproduce the Ca\textsuperscript{2+} data measured with the used experimental conditions. Below we present the results obtained by combining the Fura-2AM measurements and computational simulations.

Effects of 5-HT on the levels of cytosolic Ca\textsuperscript{2+}

When the experiments were performed in solutions with normal external Ca\textsuperscript{2+}, the addition of 5-HT every time induced a transient peak together with a more sustained increase in \([Ca^{2+}]_{cyt}\) (Figures 3A and 4). When a lesser amount of 1 \textmu M 5-HT was added for 20 seconds in Ca\textsuperscript{2+} free medium, a single peak was seen, indicating release of Ca\textsuperscript{2+} from intracellular stores (Figure 3B). The simulation of this is seen in Figure 3D. In Figures 3C and 3D, one realization of the chemical Langevin equation is printed in gray while the black traces represent the mean and standard deviations for 1000 realizations. The lack of possible differences between glutamate and 5-HT receptor activation in these cells. The ratio of emissions is directly correlated to cytosolic Ca\textsuperscript{2+} concentration \([Ca^{2+}]_{cyt}\). A deterministic model, introduced by Di Garbo et al. [31], was used as a reference model to which stochasticity was introduced by CLE in aim to reproduce the Ca\textsuperscript{2+} data measured with the used experimental conditions. Below we present the results obtained by combining the Fura-2AM measurements and computational simulations.

The model simulation closely resembled the experimental peak, except the peak duration was found to be shorter in simulations than in experiments (compare Figures 3B and 3D). With external Ca\textsuperscript{2+} present, the simulation (illustrated in Figure 3C) shows a sustained
component which, however, is shorter than seen in the experiments (compare Figures 3A and 3C).

Effects of Aβ25–35 on the basal levels of cytosolic Ca²⁺

Our earlier studies [16] showed that only 36% of astrocytes responded to Aβ25–35 additions by transient increase in [Ca²⁺]cyt, which returned back to baseline level after 1–4 minutes. In the present study, the mean value for the baseline [Ca²⁺]cyt in control astrocytes was 2.39±0.40 (mean in ratio 340/380 units ± s.d.; n = 32), and 2.69±0.60 (n = 8) in those astrocytes which were similarly cultured and then incubated with 200 nM Aβ25–35 for 40 h (see the baseline at t = 0 … 100 s in Figure 2). There is no significant difference in the baseline values of the control and Aβ25–35-treated astrocytes (p = 0.098, which is > 0.05; statistics were made using Anova module, Statistica, Statsoft Inc.), indicating that Aβ25–35 does not cause persistent change in the basal level of calcium in these cells.

Synergistic effects of Aβ25–35 and transmitters on the levels of cytosolic Ca²⁺

The mean amplitude of [Ca²⁺]cyt increase with simultaneous addition of 5-HT and Aβ25–35 was statistically significantly different (p < 0.001) from the amplitude when 5-HT alone was added. 100% (n = 43) of studied astrocytes, with or without Aβ25–35 present, responded to 10 µM 5-HT with a transient peak of increased [Ca²⁺]cyt. Aβ25–35 addition did not significantly change the mean duration or time constant of the first Ca²⁺ peak, but increased the peak amplitude, which reflects the magnitude of Ca²⁺ release from intracellular stores (compare traces in Figures 4 and 5). 1 µM Aβ25–35, when added simultaneously with 10 µM 5-HT, caused a significant 163% increase in the mean Ca²⁺ peak amplitude (n = 5) from the control value of [Ca²⁺]cyt induced by 5-HT alone (n = 13). A lesser 75% increase was detected in cells incubated with 200 nM Aβ25–35 for 48 h prior to adding 5-HT (n = 6, Figure 2). Astrocytes were also incubated with 10 nM Aβ25–35, but the detected 5-HT-induced changes in [Ca²⁺]cyt were then not significantly different from the control values.

Glutamate has earlier been shown to induce increase in intracellular Ca²⁺ [17], and also in the present study 50 µM glutamate induced increase in intracellular Ca²⁺ in 25% (n = 4) out of 16 astrocytes. Incubation of astrocytes with 10 nM or 200 nM Aβ25–35 for 48 h increased the number of cells responding to glutamate to two cells out of four tested. When Aβ25–35 was added simultaneously with glutamate, 100% (n = 6) of astrocytes responded with a Ca²⁺ increase (data not shown). Furthermore, this study revealed another interesting interaction between intracellular Ca²⁺ and glutamate: glutamate seems to be able to decrease [Ca²⁺]cyt, which has first been elevated by 5-HT, and to inhibit the Ca²⁺-oscillations and return the Ca²⁺ levels close to baseline (Figures 4, 5, and 6A). Glutamate may thus be able, by activating separate metabotropic receptors, to both increase [Ca²⁺]cyt via release from intracellular stores and influx through L-type Ca²⁺ channels, and inhibit the Ca²⁺ channel-mediated Ca²⁺ influx and oscillations. This phenomenon was seen in every cell tested (in 8 control astrocytes, 10 astrocytes incubated with 10 or 200 nM of Aβ25–35, and 12 astrocytes where Aβ25–35 had been added simultaneously with glutamate). These effects of glutamate were not included in the computational model and synergistic effects of 5-HT and glutamate with Aβ peptide fragments require further testing.

The importance of intracellular Ca²⁺ stores in Ca²⁺ signaling

The ability of recurrent additions of the transmitter to induce a Ca²⁺ release from intracellular stores was tested using different frequencies of stimuli. If the stimuli (simultaneous addition of Aβ25–35 and 5-HT in experimental measurements) were given to the system more frequently, the peak amplitudes of the latter measurements were lower. This indicated the incomplete recovery from the desensitization of the receptor or the inadequate filling of the intracellular Ca²⁺ stores between the stimuli. However, the more sustained components, originating from Ca²⁺ flux through plasma membrane, were similar, regardless of the frequency of stimuli. Simulations run with less/frequent stimuli mimicked the experimental measurements (compare Figures 6A, 6B and 7). Thus, the Ca²⁺ responses in simulations indeed depend on the

Figure 4. Changes in cytosolic Ca²⁺ concentration induced by 5-HT, glutamate, and Aβ25–35 given at different times. Small change in cytosolic Ca²⁺ concentration is seen when 10 µM 5-HT is added at t = 180 s. 50 µM glutamate reduces the 5-HT-induced enhancement of cytosolic Ca²⁺ concentration at t = 700 s, whereas additions of 1 µM Aβ25–35 do not show an increment to cytosolic Ca²⁺ concentration at t = 1050, 1100, and 1130 s.

Figure 5. Synergistic effects of 5-HT and Aβ25–35 on cytosolic Ca²⁺ concentration. Substantial change in cytosolic Ca²⁺ concentration due to synergistic effect of 10 µM 5-HT and 1 µM Aβ25–35 added at t = 180 s. 50 µM glutamate, added at t = 350 s, reduces the enhancement of cytosolic Ca²⁺ concentration.
preceding events. The more sustained component of Ca\(^{2+}\) release seemed to remain both in the experimental results and simulations, regardless of the frequencies of the stimuli. In Figures 6B and 7, one realization of the chemical Langevin equation is printed in gray while the black traces represent the means and standard deviations for 1000 realizations.

**Figure 6.** Effects of low stimulus frequency on cytosolic Ca\(^{2+}\) concentration; Fura-2AM measurements (A) and computational simulations (B). A. 1 \(\mu\)M Aβ25–35 and 10 \(\mu\)M 5-HT are added together at \(t = 150\) s and \(t = 940\) s. Glutamate is added at \(t = 300\) s and \(t = 1100\) s. The interval between the external stimuli is long enough to enable the intracellular Ca\(^{2+}\) stores to fill up between the stimuli. Thus, the peak amplitude of the latter peak is not lower than the preceding one. B. Simulations of changes in cytosolic Ca\(^{2+}\) concentration induced by 10 \(\mu\)M external stimuli given at \(t = 150\) s and \(t = 940\) s. Model simulations reproduces the phenomena seen in Figure 6A.

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**Figure 7.** Simulated effects of high stimulus frequency on cytosolic Ca\(^{2+}\) concentration. Model simulation of changes in cytosolic Ca\(^{2+}\) concentration induced by external stimuli. 10 \(\mu\)M stimuli, applied with a short interval at \(t = 115\) s and \(t = 315\) s, decrease the peak amplitude of the latter peak.

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**Discussion**

One of the hallmarks of AD are the neuritic Aβ plaques. It is still an unresolved question how Aβ fragments start to form aggregates and at what concentrations they begin to affect the cellular interactions in the brain. We have here shown that even small amounts of Aβ25–35 fragments in the rat cortical astrocytes can, together with 5-HT and glutamate, induce meaningful changes in the intracellular Ca\(^{2+}\) concentration. Aβ25–35 together with 5-HT caused an enhanced first peak of intracellular Ca\(^{2+}\) representing the release from intracellular stores mediated by 5-HT2A receptor. The glutamate induced increase in Ca\(^{2+}\) release from stores would most probably be mediated by a Group I (type 1 or 5) mGluR found in cortical astrocytes [17]. The observed additional inhibitory effect of glutamate could be the result of the activation of the Group II metabotropic glutamate receptors which are known to reduce the voltage-sensitive Ca\(^{2+}\) currents and be potential targets for neurological disorders (see [47,48]).

In this study, we used data and a computational model to characterize the Ca\(^{2+}\) transients associated with synergistic effects of Aβ25–35 and transmitter 5-HT in rat cortical astrocytes. To our knowledge, this is the first such study. In the experimental part of this study, it was shown that 5-HT and Aβ25–35, when added together, clearly increased the amplitudes of the Ca\(^{2+}\) signals. Addition of Aβ25–35, 5-HT, or glutamate alone was not able to induce that several-fold increment to the intracellular Ca\(^{2+}\), which was seen when Aβ25–35 and 5-HT were added together. The abnormal increase in intracellular Ca\(^{2+}\) may in its turn trigger a complex cascade of a variety of molecular events in the intracellular signaling pathways [16,17,49–53]. The measured Ca\(^{2+}\) signals indicate the activation of 5-HT2A receptor followed by G protein, PLC, and IP\(_3\) mediated Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. An additional Ca\(^{2+}\) influx through voltage-sensitive and -insensitive Ca\(^{2+}\) channels might be involved, as presented in [27]. Changes in astrocytic Ca\(^{2+}\) signaling are prone to cause widespread alterations in neuronal network function and can lead to neurological disorders (reviewed in [54]).

In the computational part of this study, a mathematical model by Di Carbo et al. [31], for simulating intracellular Ca\(^{2+}\) processes, was selected to be the basis for developing a more adequate model. Other models presenting Ca\(^{2+}\) signaling in astrocytes (such as in [55]) include the flux of Ca\(^{2+}\) from/to ECM, pumping Ca\(^{2+}\) to ER, and Ca\(^{2+}\) release from ER. The model selected for the present study includes six components which affect the intracellular Ca\(^{2+}\) concentration: 1) Ca\(^{2+}\) leak from/to ECM, 2) capacitive Ca\(^{2+}\) entry from ECM, 3) Ca\(^{2+}\) entry via ionotropic receptors, 4) Ca\(^{2+}\) leak from intracellular stores, such as ER, 5) storage of Ca\(^{2+}\) to ER via SERCA pumps, and 6) Ca\(^{2+}\) release from ER mediated by IP\(_3\). Due to different experimental setups, some of the components in the six-component model had to be restrained. The hypothesis about Ca\(^{2+}\) liberation from the intracellular stores was first experimentally verified, and then reproduced by simulations. The simulations supported the experimental findings in both Ca\(^{2+}\) free media and with normal extracellular Ca\(^{2+}\) containing environment. The variability of biological signals cannot be accurately mimicked by deterministic models alone, which justified the use of stochastic methods.

A mathematical model, presented in this study, integrates data from several experimental sources and thus provides a way to computationally follow Ca\(^{2+}\) changes in biologically relevant conditions. Here, the stochastic model was able to reproduce the Ca\(^{2+}\) signals seen in the experimental Fura-2AM measurements.
Potential pitfalls of modeling, in general, is the inadequate experimental data. Experiments should originally be designed also to fulfill the demands of a modeling approach, including the need of considerable amount of repetitions, relevant statistics, and adequate metadata. When new components, describing cellular functions, will be added in the model, it will help to explore further the possible mechanisms behind the measured Ca$^{2+}$ signals. This may advance the study of astrocytic Ca$^{2+}$ signals and their effects on neuronal networking in the central nervous system, by adding information of the intracellular targets activated by Ca$^{2+}$ transients [studies on astrocytic Ca$^{2+}$ waves are reviewed in [56]]. Calcium transients are known to affect the important intracellular Ca$^{2+}$ sensitive peptides, such as protein kinases and phosphatases. In addition, the passage of Ca$^{2+}$ signals could lead to the priming of the astrocytes, thus modifying forthcoming astrocytic responses, setting the cellular basis for plasticity in glial cells [56]. Leisring et al. [57] have discussed the possibility that mutations in presenilin 1 (one of the factors in familial AD involved in the accumulation of amyloid β fragments in the brain) may change the activity of the ER Ca$^{2+}$-ATPases, e.g., SERCA. ATPases are associated with pumping the cytosolic Ca$^{2+}$ into the ER lumen, leading eventually to higher concentration of Ca$^{2+}$ in ER. Amyloid β peptide accumulation may lead to higher-amplitude [Ca$^{2+}$]$_{cyt}$ signals, have an effect on other Ca$^{2+}$-induced release, and increase intracellular IP$_3$ sensitivity [57]. Thus, the exceptional cytosolic Ca$^{2+}$ signals via ER, overfilled with Ca$^{2+}$, may explain the Ca$^{2+}$ changes detected in the familial AD. Possible extension of the here developed stochastic model could be the incorporation of some specific IP$_3$R model into the proposed model to study the role of altered IP$_3$ sensitivity on the overall Ca$^{2+}$ signaling. The simulations run with our stochastic model did not take into account the possibility that the synergistic effects of Aβ25–35 and 5-HT could be due to increased activation of, e.g., SERCA pumps. In addition, the pitfall of the here introduced stochastic model is that it does not take into account spontaneous Ca$^{2+}$ signaling in astrocytes (modeled, e.g., in [55]). To include these phenomena into our stochastic model would need further studies and tuning of model parameters. Progressive inclusion of additional components could lead to a still more realistic model of the Ca$^{2+}$-signaling in astrocytes. In general, a better understanding of the involvement of astrocytes in the developing pathology of Alzheimer’s disease is of great importance for the future development of diagnosis and treatment. Early diagnosis of AD is important for initiating treatment and for understanding the pathobiology of the disease [58]. Aβ-induced astrocyte activation is thought to have a critical role in the mechanisms of neurodegeneration in AD [59], as astrocytes signal to neurons in response to a physiological stimulus (see, e.g., [60]). The active participation of astrocytes in synaptic processes is of utmost importance for physiology of the nervous system [61,62]. Studies combining experimental and computational experiments, like the present one, are required as they may provide us novel viewpoints and help explaining the possible mechanisms behind certain experimental findings.

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Author Contributions
Conceived and designed the experiments: ET TM TJ ML. Performed the experiments: ET TM AN TJ. Analyzed the data: ET TM TJ. Wrote the paper: ET TM TJ ML.

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