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Research Article

Short-Term Amyloid Beta Application Decreased Glutamate Release, but Increased Glutamate Spillover in Hippocampal Neurons

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Abstract

Aim: Synaptic dysfunction is a characteristic linked with the early stages of Alzheimer's disease (AD), but the pathological mechanisms remain elusive. It was aimed to investigate how amyloid beta 42 (Abeta42) peptide affects miniature events mediated by glutamate release in hippocampal neurons.

Material and Methods: We performed all experiments in the primary cultured hippocampal neurons in control and Abeta42-treated neurons (24 h). Pharmacologically isolated miniature excitatory postsynaptic currents (mEPSCs) were obtained in whole-cell voltage-clamp configuration at – 70 mV. AMPAergic channel conductance and basic synaptic parameters were evaluated by performing peak-scaled variance analysis and cumulative event analysis and glutamate spillover is determined by application of DNQX.

Results: The oligomeric Abeta42 for 24h decreased the mEPSCs frequency (***p<0.001), while it has no any measurable effect on the amplitude of mEPSCs as well as unitary current and number of receptors. In addition, the incubation of neurons with oligomeric Abeta42 for 24h increased the glutamate spillover measured as baseline shift (***p<0.001).

Conclusion: The oligomeric form of the Abeta42 peptide has a significant effect on the presynaptic site of excitatory synapses in primary cultured hippocampal neurons. It lowers the release probability during short-term incubation, while it increases glutamate spillover.

Keywords: AMPAergic synapses, amyloid beta peptide, Alzheimer's disease, glutamate spillover

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that predominantly affects the elderly population and is characterized by a gradual decline in cognitive function, memory loss, and behavioural changes (1). One of the key pathological hallmarks of Alzheimer's disease is the accumulation of abnormal protein aggregates, including amyloid beta peptide and tau tangles, within the brain (2-4). While these proteinopathies have long been central to understanding the disease, emerging research has highlighted the critical role of the amyloid beta 42 (Abeta42) peptide on synaptic dysfunction and thus the development and progression of AD, since the amyloid beta accumulation precedes the hyperphosphorylated tau tangles (5,6).

Synapses, the specialized connections between neurons, play a fundamental role in transmitting and processing information in the brain. Proper synaptic function is essential for various cognitive processes, including learning, memory, and information processing. However, in AD, synaptic dysfunction emerges as a pivotal contributor to the cognitive decline observed in patients and animal models (7,8). The disruption of synaptic connections and neurotransmitter imbalances impair neural communication, leading to the cognitive impairments associated with the disease.

In this study, we aimed to determine the relationship between the early pathology of AD and excitatory synaptic dysfunction, exploring how the accumulation of Abeta42 peptides contribute to synaptic alterations, specifically excitatory synapses mediated by AMPA receptors. By understanding the cellular mechanisms underlying these possible synaptic alterations, we also aimed to

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uncover potential therapeutic targets that could mitigate cognitive decline and offer new avenues for managing this devastating neurological disorder.

MATERIAL AND METHOD

Cell Culture

To harvest tissues, animals were humanely euthanized by gradual exposure to increasing levels of CO_2 followed by swift cervical dislocation. The 18-day-old embryos of C57BL6 mice (Envigo, San Pietro al Natisone, Italy) were used to obtain hippocampal neurons. They were promptly retrieved through caesarean section and swiftly decapitated before extraction. Under sterile conditions, the rapidly dissected hippocampus was placed in cold high-glucose HBSS (4°C), and then subjected to digestion using papain (0.5 mg/mL) supplemented with DNAse (0.1 mg/mL) (9). Subsequently, the isolated neurons were plated at a density of 100 cells/mm² and cultivated in a medium composed of Neurobasal B-27 (at a 1:50 v/v ratio), 1% w/v glutamine, and 1% penicillin-streptomycin. A semi-weekly half-volume medium exchange was conducted over a span of 2-3 weeks.

Solutions and Drugs

To record miniature excitatory postsynaptic currents (mEPSCs), the extracellular solution employed was Tyrode's standard solution, comprising the following concentrations (in mM): 2 $CaCl_2$, 130 NaCl, 2 $MgCl_2$, 10 HEPES, 4 KCl, 10 glucose, with a pH of 7.4. The internal solution was composed of the following concentrations (in mM): 135 gluconic acid (potassium salt: K-gluconate), 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 2 ATP-Tris, and 0.4 Tris-GTP. To inhibit NMDA, GABAA, and GABAB receptors, respectively, d-(−)-2-Amino-5-phosphonopentanoic acid (d-AP5; 50 µM, Sigma-Aldrich), picrotoxin (100 µM, Sigma-Aldrich), and CGP58845 (5 µM, Tocris) were used. To prevent spontaneous action potential propagation, 300 nM tetrodotoxin was added into the external solution. 6,7-dinitroquinoxalone-2,3-dione (DNQX, 20 µM) was used to evaluate glutamate spillovers. Abeta42 obtained from Sigma-Aldrich was solubilized in a solution of dimethyl sulfoxide (DMSO) and then stored at a temperature of -20°C, a concentration of 1mM. As previously described (10), the hippocampal cultures were exposed to Abeta42 (1 µM) for 24 hours before the performing of the electrophysiological experiments.

Patch Clamp Experiments

Borosilicate glass (Hilgenberg, Mansfield, Germany) were used to obtain patch electrodes (3 to 5 MΩ). All measurements were executed in the whole-cell configuration. The mEPSCs were recorded from 22-day in vitro (22 DIV) primary cultured hippocampal neurons (22-24°C) at −70 mV (Vh). The mEPSCs were recorded at a sampling rate of 10 kHz (Bessel filter set at 1 kHz). The analysis of frequency and amplitude of mEPSCs was performed with Clampfit Software (Molecular devices). Mini Analysis program (Synaptosoft, Leonia, NJ, USA) was used to noise analysis.

Non-stationary noise analysis of mEPSCs was conducted, taking into account the parabolic relationship between the variance (σ^2) of current and the mEPSC amplitude (11). In order to separate current fluctuations attributed to the stochastic characteristics of channel gating from those resulting from variations in neurotransmitter release and the quantity of postsynaptic receptors, the average waveform derived from the mEPSCs was normalized to the peak of each individual mEPSC. The unitary current was estimated by fitting the relationship between peak-scaled variance $\sigma^2(t)$ and the mean amplitude I(t) with the following equation: $\sigma\ 2(t)=iI(t)-(I^2(t)/Nch)+\sigma B^2$, where i is the weighted mean unitary current and Nch is the number of channels activated by a signle quantum of vesicle release.

Glutamate spillover was estimated by application of AMPA receptor antagonist, 6,7-dinitroquinoxalone-2,3-dione (DNQX, 20 µM), and measured as baseline shift.

Statistical Analysis

The data are presented as mean±S.E.M. for a total of n cells. The normal distribution of data was evaluated using the D'Agostino Pearson's normality test. Unless otherwise indicated, the statistical significance has been assessed by considering two sample groups that are normally distributed, and the unpaired Student's t-test was applied. In instances where the two sample groups were not normally distributed, we employed the non-parametric Kolmogorov-Smirnov test. Data were considered statistically significant when $p<0.05$.

RESULTS

To investigate the presynaptic and postsynaptic effects induced by Abeta42 in AMPAergic synapses, we firstly focused on the miniature excitatory postsynaptic currents (mEPSCs) in primary cultured hippocampal neurons in in vitro day (DIV) of 22-23 in control and neurons of pretreated with the Abeta42 for 24 h (Figure 1a for WT and Abeta42, respectively).

With 24 h-incubation of neurons with oligomeric Abeta42, it was significantly rightward shifted the inter-event intervals (IEIs) of mEPSCs (Figure 1b) (***$p<0.0001$, Kolmogorov-Smirnov test), but has no any measurable effect on the amplitude of mEPSCs (Figure 1c). In fact, the mean of mEPSCs IEI (Figure 1b, inset bar graph) increased from 289.7±45.08 ms in ctrl to 478.0±48.96 ms in Abeta42-treated neurons for 24 h (n=9 cells for ctrl and Abeta42-treated neurons), whereas it has no any measurable effects on the mEPSCs amplitude (Figure 1c, inset bar graph) (15.86±0.64 pA in ctrl and 17.29±0.56 pA in Abeta42-treated neurons for 24 h (n=10 cells for ctrl and Abeta42-treated neurons) ($p>0.05$). We then evaluated how Abeta42 affects the number of AMPA receptors (AMPARs) and its single channel current through peak-scaled variance analysis (11). We found that the parabolic relationship between variance (σ^2) and mean mEPSCs amplitude were not affected by the Abeta42 (Figure 2a and b), observing unchanged steepness and width of parabolic fit. With

the result of the unchanged steepness and its width, it is possible say that the Abeta42 does not have any effect on the unitary current of AMPARs (1.14±0.09 pA in ctrl and 1.23±0.07 pA in Abeta42-treated neurons for 24 h) (n=10 cells for ctrl and Abeta42-treated neurons, p>0.05, Unpaired Student's t-test) (Figure 2c) and the number of AMPARs which is activated by one vesicle release (13.81±0.96 in ctrl to 15.36±1.25 in Abeta42-treated neurons) (n=10 cells for ctrl and Abeta42-treated neurons, p>0.05, Unpaired Student's t-test) (Figure 2d), respectively. These results imply that oligomeric Abeta42 has an initial impact only on the presynaptic site by reducing release probability, without affecting postsynaptic site of excitatory neurons in primary cultured hippocampal neurons.

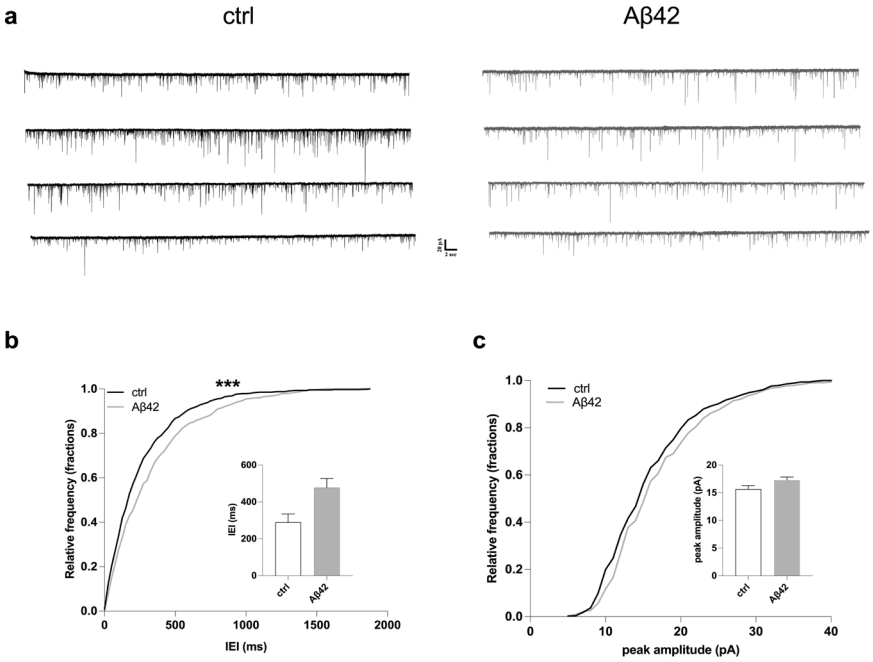

Figure 1. Abeta42 lowers the frequency of AMPAergic mEPSCs in the hippocampal neurons, **1a.** representative mEPSCs measured from four different cells in ctrl (left, black) and in Abeta42 treated neurons (right, grey), **1b.** and **1c.** cumulative distribution of mEPSCs inter-event interval (IEIs) and amplitude measured in ctrl (black) and Abeta42-treated neurons (grey) (***p<0.001). Inset bar graphs demonstrate the mean IEI and amplitude of mEPSCs in ctrl (black) and in the Abeta42-treated neurons (grey)

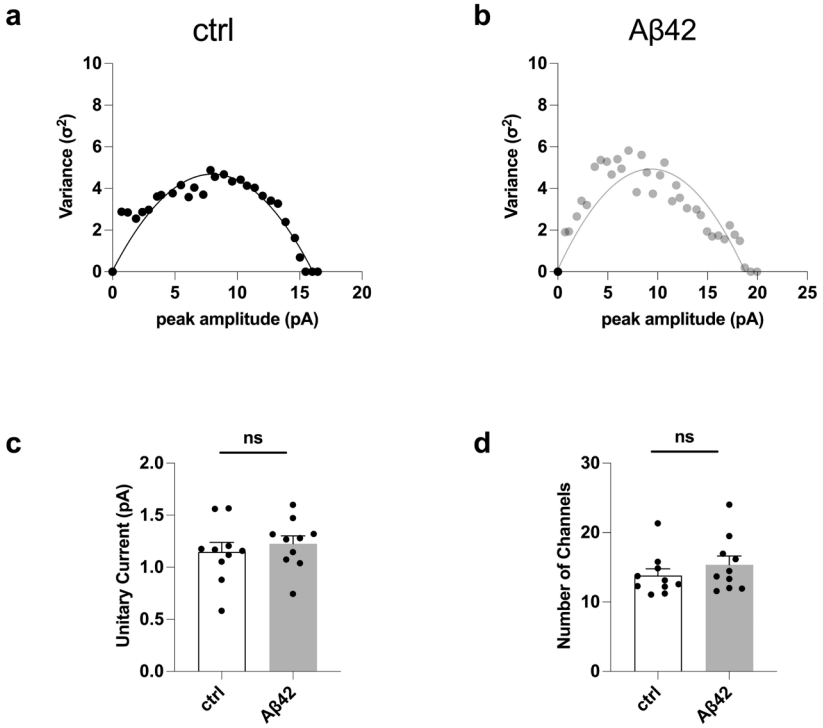

Figure 2. Abeta42 does not have any effect on the mean unitary current and number of receptors. a and b, parabolic fit of mEPSC variance (σ2) as a function of their amplitude in ctrl **2a.** and Abeta42-treated neurons **2b.** c and d, the parabolic fit provides information about unitary current **2c.** and number of AMPARs **2d.** (p>0.05)

Lastly, we investigated glutamate spillover by application of 20 μM DNQX (Figure 3a, ctrl and Abeta42-treated neurons, respectively). The results showed that oligomeric Abeta42 incubation for 24h induced a significant increase in the tonic excitation mediated by glutamate spillover (9.90±2.13 pA in ctrl vs. 24.29±4.66 pA in Abeta42-treated neurons) (n=10 cells for ctrl and Abeta42-treated neurons, P>0.05, Unpaired Student's t-test) (Figure 3b). This result indicated that oligomeric Abeta42 peptide has an early effect on the glutamate reuptake mechanism.

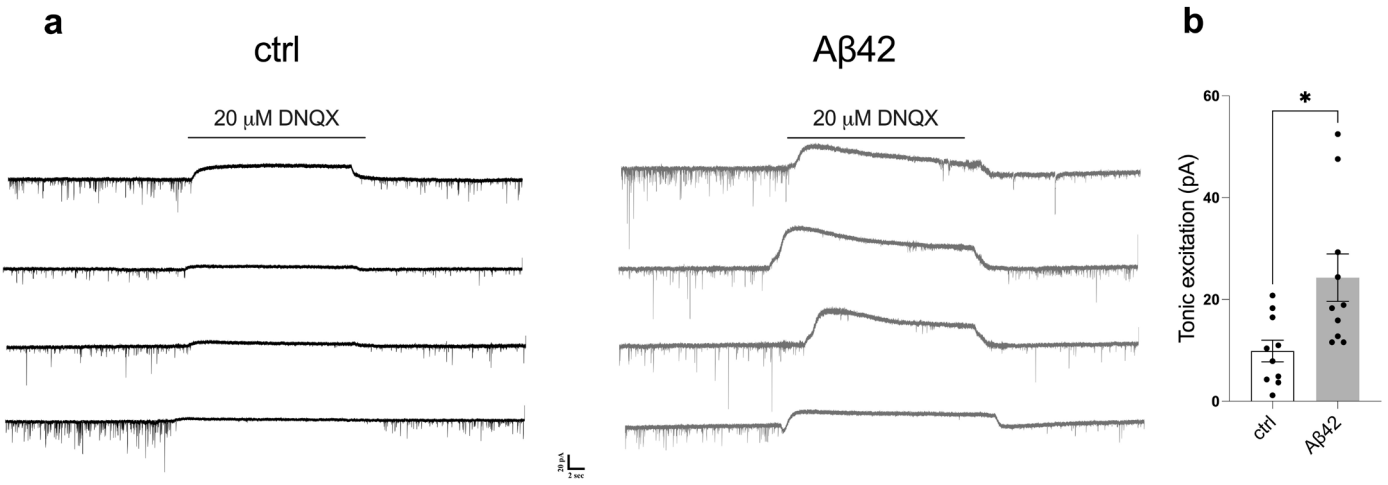

Figure 3. Abeta42 induces an increase in tonic excitation, indicating an augmentation in glutamate spillover, **3a.** representative mEPSCs recorded from four different cells in ctrl (left, black) and Abeta42-treated neurons (right, grey) with 20 μM DNQX, **3b.** bar graph summarizing the average tonic excitation measured by application of 20 μM DNQX (*p<0.05)

DISCUSSION

The hippocampus is a critical brain region located in the temporal lobe and composed of diverse array of neurons, including both excitatory and inhibitory neurons, which plays a pivotal role in the formation, consolidation and retrieval of memories as well as spatial navigation. In AD, the hippocampus is among the earliest and most severely affected brain regions and hippocampal overexcitability is observed during early stages of AD pathology. The underlying cause of this hyperexcitability is thought to be due to pathological changes in the glutamatergic system and reducing neuronal excitability might provide a potential therapeutic avenue for AD, but the exact mechanisms still remain unclear.

Previous studies suggest that accumulation of Abeta peptide can modulate the efficacy of excitatory neurotransmission by affecting the availability of synaptic vesicles, neurotransmitter release probability, and the function of postsynaptic receptors. These alterations may contribute to aberrant synaptic signaling and potentially contribute to cognitive impairments observed in AD. While the alterations in the excitatory system caused by the accumulation of Abeta are undoubtedly established, conflicting findings have emerged from various studies-likely attributed to differences in experimental models, time duration of incubation and oligomerization process of Abeta peptide. In hippocampal neurons, it has been observed that the release probability is significantly decreased following acute application of or incubation of neurons with Abeta42 peptide (<1μM) for 24 hours. For example, He. et al. (2019) have reported that both the frequency and the amplitude of mEPSCs are significantly decreased following application of 400 nM Abeta42 in the hippocampal neurons, suggesting reduced release probability. They also provided evidence that this type of reduction observed in release probability is mainly due to the presynaptically phosphatidylionositol-4-5-biphosphate (PIP2) depletion in axons (12). On the other hand, the hippocampal neurons, exposed for 24 h to various concentrations (from 5 nM to 10 μM) of Abeta, have exhibited different effects on the excitatory synaptic transmission in cultured hippocampal neurons. The high concentrations of Abeta, ranging from 50 nM to 1 μM, exhibited a reduction in the frequency of mEPSCs. However, this effect was absent when Abeta was present in low concentration (5 nM), indicating no influence on synaptic transmission. A separate investigation in this study noted that distinct variations of Abeta (monomers and fibrils) did not yield any significant effects on excitatory synaptic transmission (13). Our results are partly consistent with these studies except of the effects of Abeta42 on the amplitude of mEPSCs. In our study, we demonstrated a significant decrease in the frequency of mEPSCs following a 24-hour incubation of hippocampal neurons with oligomeric form of Abeta42 peptide, whereas no observable changes were detected in the amplitudes of these events. In addition, we did not observe any significant effects of oligomeric Abeta42 peptide on the single channel conductance and the number of AMPARs. These outcomes suggest that, under in vitro conditions, the primary influence of the oligomeric Abeta42 peptide pertains to the presynaptic site, without affecting the postsynaptic site.

Nevertheless, these findings appear incongruent with the increased neuronal activity reported in existing literature (14-16). One potential explanation for these contradictory results is that many studies reporting hyperexcitability involved prolonged Abeta exposure, disregarding its earliest effect, which entails a decline in glutamate release. The effects induced by the application of Abeta42 peptide for 48 hours were also comparable in excitatory synapses mediated by NMDA receptors and the authors have reported that Abeta42 activates synaptic responses mediated by NMDA through intracellular calcium release from internal stores through ryanodine receptors (RyRs) in the the early stages of AD (17). However, in this study, we observed that tonic excitation was increased by application of oligomeric form of Abeta42, suggesting that glutamate reuptake rate is decreased in early stages of amyloid pathology. This results with the overaccumulation of glutamate in synaptic cleft, inducing hyperexcitability in excitatory neurons. Considering all these findings, it is reasonable to conclude that the accumulation of Abeta42 peptide primarily affects presynaptic site by reducing glutamate release as well as reuptake rate in excitatory synapses. The limitation of this study is that glutamate reuptake mechanisms were not studied in detail. Therefore, in future studies, it is necessary to investigate the glutamate reuptake mechanisms and to what extent they are affected by Abeta pathology.

CONCLUSION

In conclusion, we here demonstrated that the oligomeric form of the Abeta42 peptide elicits presynaptic effects, leading to a reduction in glutamate release and it also disrupts glutamate reuptake mechanism on excitatory synapses in primary cultured hippocampal neurons. These findings highlight that a mechanism that can regulate the glutamate release and reuptake mechanism observed in the early stages could be an effective treatment method for AD pathology.

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Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *All experimental protocols received ethical approval from the University of Turin Animal Care and Use Committee in Turin, Italy and adhered to the National Guide for the Care and Use of Laboratory Animals as outlined by the Italian Ministry of Health (Authorization 695/2020-PR). The animals were provided with unrestricted access to water and food within their shelter. Every possible measure was taken to mitigate animal distress and limit the utilization of animals.*

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