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Early Alterations of Hippocampal Neuronal Firing Induced by Abeta42

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Early alterations of hippocampal neuronal firing induced by Abeta42

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3 Early alterations of hippocampal neuronal firing induced by Abeta42
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23 Running title: Neuronal dysfunctions induced by Abeta42 oligomers
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Abstract

We studied the effect of Amyloid β 1-42 oligomers (Abeta42) on Ca^{2+} dependent excitability profile of hippocampal neurons. Abeta42 is one of the Amyloid beta peptides produced by the proteolytic processing of the amyloid precursor protein (APP) and participates in the initiating event triggering the progressive dismantling of synapses and neuronal circuits. Our experiments on cultured hippocampal network reveal that Abeta42 increases intracellular Ca^{2+} concentration by 46% and inhibits firing discharge by 19%. More precisely, Abeta42 differently regulates ryanodine (RyRs), NMDA receptors (NMDARs) and voltage gated calcium channels (VGCCs) by increasing Ca^{2+} release through RyRs and inhibiting Ca^{2+} influx through NMDARs and VGCCs. The overall increased intracellular Ca^{2+} concentration causes stimulation of K^+ current carried by big conductance Ca^{2+} activated potassium (BK) channels and hippocampal network firing inhibition. We conclude that Abeta42 alters neuronal function by means of at least four main targets: RyRs, NMDARs, VGCCs and BK channels. The development of selective modulators of these channels may in turn be useful for developing effective therapies that could enhance the quality of life of AD patients during the early onset of the pathology.

Keywords: big conductance Ca^{2+} activated potassium (BK) channels, NMDA receptors, ryanodine receptors, spontaneous calcium transients, voltage gated Ca^{2+} channels.

Introduction

Among the various hallmarks of Alzheimer's disease (AD), the activation process of the "amyloid-cascade" is one of the most studied. It assumes that the accumulation of oligomers of Amyloid Beta peptides (Abeta), produced by the proteolytic processing of the amyloid precursor protein (APP) is the initiating event that triggers the progressive dismantling of synapses, neuronal circuits and networks (Palop JJ and L Mucke 2010). However, so far there are not yet clear data regarding any possible Abeta-induced impairment of neuronal network excitability. Our previous results indicate that in Tg2576 mice neurons from lateral entorhinal cortex (LEC) (Marcantoni A et al. 2014) are characterized by early impairments of their excitable profile. Tg2576 mice harbor the human APP gene with the Swedish mutation and display age-dependent accumulation of Abeta peptides, like Abeta40, Abeta42, Abeta*56 and exhibit hyperphosphorylated tau (Hsiao K et al.

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3 1996). The use of these mice does not therefore clarify if one or more Abeta peptides cause
4 neuronal dysfunctions. These latter are generated by multiple interactions between Abeta
5 oligomers and neurons that could occur by their direct binding to membrane receptors, interaction
6 with membrane lipids leading to pore channels formation or changes of membrane properties
7 (Benilova I et al. 2012). The manifold mechanisms of interplay between Abeta peptides and
8 neurons are followed by variable effects on neurons and vary from synaptic plasticity impairment
9 (Puzzo D et al. 2015) to spines and cells loss. Here we propose to focus on the effects of Amyloid
10 β 1-42 oligomer (Abeta42), whose accumulation induces early and severe neuronal function
11 impairments (Lambert MP et al. 1998; Ripoli C et al. 2013). There are however contrasting findings
12 concerning the effect of Abeta42 on neuronal excitability and synaptic function. Recent evidences
13 show that spontaneous firing of cultured neuronal network recorded by means of microelectrode
14 arrays (MEAs) is depressed by Abeta42 (Charkhkar H et al. 2015), that in turn inhibits synaptic
15 function (Ripoli C et al. 2013). Other evidences indicate that Abeta42 enhances neuronal
16 excitability (Minkeviciene R et al. 2009; Puzzo D et al. 2009; Tamagnini F et al. 2015). All together
17 these results suggest that the effect of Abeta42 strongly depends on its concentration and
18 aggregation state (Palop JJ and L Mucke 2010), but they fail to clarify its mechanism of action.
19 Likewise, the existence of a correlation between excitatory impairments and Ca^{2+} dysregulation
20 induced by Abeta42 is still not completely understood. Ca^{2+} regulates many neuronal functions
21 such as gene transcription, excitability and synaptic activity. It is generally accepted that during AD
22 both the mechanisms of Ca^{2+} entry (Thibault O et al. 2012; Wang Y and MP Mattson 2013) and
23 Ca^{2+} release (Stutzmann GE et al. 2004; Oules B et al. 2012) are altered. This is shown both in
24 transgenic mice (Oules B et al. 2012; Chakroborty S et al. 2013) and in neurons treated with Abeta
25 peptides (Mattson MP 2010) as well as Abeta42 (Lazzari C et al. 2014). Here we studied the effect
26 of Abeta42 on the Ca^{2+} dependent excitatory profile of hippocampal neurons using MEAs
27 extracellular recordings on neuronal circuits, intracellular patch clamp recordings and Ca^{2+}
28 fluorescence imaging on single neurons. When maintained in primary culture, hippocampal
29 neurons generate spontaneous bursts of action potentials (Gavello D et al. 2012; Allio A et al.
30 2015) that occur in synchrony with phasic periods of Ca^{2+} entry (Murphy TH et al. 1992). These
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3 neurons are in fact connected by synaptic contacts morphologically and physiologically similar to
4 those present in vivo and are useful to investigate the relationship between excitability and
5 synaptic function. We found that pre-incubation of neurons with Abeta42 raises intracellular Ca^{2+}
6 concentration and causes a paradoxical firing inhibition. We suggest that Abeta42 stimulates Ca^{2+}
7 to be released through ryanodine receptors (RyRs) and inhibits Ca^{2+} influx through NMDA
8 receptors (NMDARs) as well as through voltage gated calcium channels (VGCCs). The Abeta42-
9 induced Ca^{2+} release activates presynaptic BK Ca^{2+} activated potassium channels and inhibits
10 neuronal firing by mainly acting at presynaptic glutamatergic terminals.
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19 **Material and Methods**

20 *Abeta42 preparation*

21 Abeta42 (Sigma-Aldrich, St. Louis, MO) was dissolved in 1% ammonium hydroxide solution and
22 stored at $-20^{\circ}C$ at a concentration of 1 mM. Before neuronal administration Abeta42 was dissolved
23 into the culture medium at the experimental concentration of 1 μM . For the aggregation protocol
24 the peptide was stored 24 h at $4^{\circ}C$ to obtain oligomers and 48 h at room temperature before
25 incubation with neurons (Dahlgren KN et al. 2002; Tamagno E et al. 2006). Unless indicated
26 otherwise, the results were always obtained by comparing control neurons treated with picrotoxin
27 (100 μM) for 48 h vs neurons incubated for 48 h with Abeta42 together with picrotoxin.
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37 *AFM (Atomic Force Microscopy)*

38 Measurements were performed in the intermittent-contact regime (tapping-mode AFM, pyramidal
39 cantilever with a tip radius $<6nm$) by using a modified Nanosurf Easyscan2 AFM instrument,
40 equipped with a 10 μm high-resolution scan head, shielded by an acoustically insulated enclosure
41 and placed on an anti-vibration platform. Before analysis, sample solution containing Abeta42
42 oligomers or fibrils at a concentration of 1 μM were diluted to 100 nM and then dropped on freshly
43 cleaved mica. Samples were AFM imaged as soon as supports become dry.
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52 *Cell culture*

53 All experiments were performed in accordance with the guidelines established by the National
54 Council on Animal Care and approved by the local Animal Care Committee of Turin University.
55 Hippocampal neurons were obtained from c57bl6 mouse 18-day embryos. Hippocampus was
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3 rapidly dissected under sterile conditions, kept in cold HBSS (4°C) with high glucose, and then
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5 digested with papain (0.5 mg/ml) dissolved in HBSS plus DNase (0.1 mg/ml) as previously
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7 described (Gavello D et al. 2012). Isolated cells were then plated at the final density of 1200
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9 cells/mm² onto the MEA (previously coated with poly-DL-lysine and laminine). Cells were
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11 incubated with 1 % penicillin/streptomycin, 1 % glutamax, 2.5 % fetal bovine serum, 2 % B-27
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13 supplemented neurobasal medium in a humidified 5 % CO₂ atmosphere at 37°C. Each MEA dish
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15 was covered with a fluorinated ethylene-propylene membrane (ALA scientific, Westbury, NY, USA)
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17 to reduce medium evaporation and maintain sterility, thus allowing repeated recordings from the
18
19 same chip. Recordings were carried out at DIV 18.

20 21 *Patch clamp experiments*

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23 Patch electrodes, fabricated from thick borosilicate glasses (Hilgenberg, Mansfield, Germany),
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25 were pulled to a final resistance of 3-5 MΩ. Patch clamp recordings were performed in whole cell
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27 configuration using a Multiclamp 700-B amplifier connected to a Digidata 1440 and governed by
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29 the pClamp10 software (Axon Instruments, Molecular Devices Ltd, USA).

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31 Experiments were performed at room temperature (22-24 °C) in whole cell configuration and
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33 acquired with sample frequency of 10 KHz (Baldelli P et al. 2002; Baldelli P et al. 2005). Analysis
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35 was performed with Clampfit software (Axon Instruments). Data are expressed as means ± S.E.M
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37 and statistical significance was calculated by using Student's t-test. Values of p< 0.05 were
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39 considered significant. Where indicated, analysis of variance (ANOVA) followed by Bonferroni
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41 post-hoc test was used and p values <0.05 were considered significant.

42 43 *Current clamp*

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45 Neurons were clamped at -70 mV (V_h) and depolarized for 50ms by injecting 800 pA of current.

46 47 *Voltage clamp*

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49 For Ca²⁺ current recording neurons were hold at -70 mV (V_h) and depolarized for 50ms at -10mV in
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51 order to maximally activate inward Ca²⁺ current. K⁺ currents were recorded by depolarizing
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53 neurons from -70 to +80 mV for 400 ms. Where indicated the depolarizing step was preceded by
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55 stimuli of increased duration (from 10 to 90 ms) to -10 mV to open all available voltage-gated Ca²⁺
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57 channels (Marcantoni A et al. 2010). AMPA dependent eEPSCs following spontaneous APs or
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3 electrical stimulation were recorded by holding neurons at -70 mV (V_h). Presynaptic electrical
4 stimuli were delivered through a glass pipette (1 μ m tip diameter) filled with Tyrode's solution and
5 placed in contact with the soma of presynaptic neuron in a loose-seal configuration. eEPSCs were
6 filtered at half the acquisition rate with 8-pole low-pass Bessel filter. Recording with leak current
7 >100 pA or series resistance >20 M Ω were discarded. Current pulses of 0.1 ms and variable
8 amplitude (10 – 45 μ A) were generated by an isolated pulse stimulator (model 2100; A-M Systems,
9 Carlsburg, WA, USA).

17 *MEA recordings*

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19 Multisite extracellular recordings from 60 electrodes were performed using the MEA-system,
20 purchased from Multi-Channel Systems (Reutlingen, Germany). Data acquisition was controlled
21 through MC_Rack software (Multi-Channel Systems Reutlingen, Germany), setting the threshold
22 for spike detection at -30 μ V and sampling at 10 kHz. Experiments were performed in a non-
23 humidified incubator at 37° C and with 5% CO_2 , without replacing the culture medium. Before
24 starting the experiments, cells were allowed to stabilize in the non-humified incubator for 90
25 seconds; then the spontaneous activity was recorded for 2 minutes.

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27 Culture medium was supplemented with picrotoxin (100 μ M, Sigma Aldrich) for inhibiting the
28 GABAergic synaptic currents.

37 *Analysis of MEA activity*

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39 Bursts analysis was performed using Neuroexplorer software (Nex Technologies, Littleton, MA,
40 USA) after spike sorting operations. A burst consists of a group of spikes with decreasing
41 amplitude, thus we set a threshold of at least 3 spikes and a minimum burst duration of 10 ms. We
42 set interval algorithm specifications such as maximum interval to start burst (0.17 sec) and
43 maximum interval to end burst (0.3 sec) recorded in 0.02 s bins (Gavello D et al. 2012). Burst
44 analysis was performed by monitoring the following parameters: mean frequency, number of bursts
45 and mean burst duration. Cross correlation probability values were obtained by means of
46 Neuroexplorer software using ± 2 s and 5 ms bin size.

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3 Data are expressed as means \pm S.E.M and statistical significance was calculated by using
4 Student's t-test. Values of $p < 0.05$ were considered significant. The number of experiments is
5 referred to the number of MEA each of those consisted of 59 recording electrodes.
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8 *Calcium imaging*

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10 Hippocampal neurons plated on glass petri dishes were loaded with the fluorescent Ca^{2+} indicator
11 Fura2-AM dye (3 μM) (Invitrogen, Molecular Probes, Oregon, USA) for 1 hour. The fluorescent dye
12 was then washed, and extracellular solution containing picrotoxin (100 μM) was replaced into the
13 petri dish. The inverted microscope used (Leica DMI3000B, Wetzlar, Germany) was equipped with
14 a short-arc xenon gas discharge lamp (Ushio, Cypress, California, US). Alternating excitation
15 wavelengths of 340 nm and 380 nm were obtained by a monochromator (Till Photonics, Grafelfing,
16 Germany). The emitted fluorescence was measured at 500-530 nm. Images were projected onto a
17 EMCCD camera (QuantEM:512SC, Photometrics, Tucson, Arizona, US) and stored every 200 ms.
18 Spontaneous Ca^{2+} transients were recorded both in control condition and after incubation with
19 Abeta42 using Metafluor software (Molecular Devices, California, USA) for data acquisition. The
20 relative change in fluorescence ($\Delta F/F$) was measured considering the peak of Ca^{2+} transients. Data
21 are expressed as means \pm S.E.M and statistical significance was calculated by using Student's t-
22 test. Values of $p < 0.05$ were considered significant.
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37 *Solutions and drugs*

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39 For voltage clamp recording of Ca^{2+} currents the external solution contained (in mM): 135 TEACl,
40 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, 10 glucose (pH 7.4 with CsCl). The internal solution contained (in
41 mM): 90 CsCl, 20 TEACl, 10 EGTA, 10 Glucose, 1 MgCl , 4 ATP, 0.5 GTP, 15 Phosphocreatine (pH
42 7.4 with CsOH). For Ca^{2+} current recording, 300 nM TTX (Tocris Bioscience, Bristol, UK), 1 mM
43 kynurenic acid (Sigma Aldrich), 100 μM picrotoxin were added to extracellular solution.
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49 When K^+ current recording, Tyrode's Standard solution was used as extracellular solution and the
50 intracellular solution was the same used in current clamp experiments.
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54 For current-clamp recording extracellular Tyrode's Standard solution contained (in mM) 2 CaCl_2 ,
55 130 NaCl, 2 MgCl_2 , 10 HEPES, 10 glucose, 4 KCl (pH 7.4). The intracellular solution contained (in
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3 mM): 135 gluconic acid (potassium salt: K-gluconate), 5 NaCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 2
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5 ATP-Tris, 0.4 Tris-GTP.

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7 Nifedipine and paxilline were used to block respectively L-type Ca²⁺ channels and BK channels
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9 (Marcantoni A et al. 2010) while apamine (200 nM) was used to inhibit SK channels (Vandael DH
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11 et al. 2012) All these compounds were purchased from Sigma Aldrich.

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13 eEPSCs were recorded by perfusing postsynaptic neurons with Tyrode's Standard solution
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15 supplemented with D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5; 50 μM), picrotoxin (100 μM)
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17 and CGP58845 (5 μM) (Tocris, Bristol, UK) to avoid contamination from NMDA dependent and
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19 GABAergic receptors activated currents. Action potential driven eEPSCs were recorded by adding
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21 Tetrodotoxin (10 nM) to limit polysynaptic activity (Raffaelli G et al. 2004). The standard internal
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23 solution was (in mM): 20 Cs-MSO₃, 90 CsCl, 10 Hepes, 5 EGTA, 2 MgCl₂, 4 ATP (disodium salt),
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25 15 Phosphocreatine (pH 7.4). Electrically stimulated eEPSCs were recorded by adding QX-314 (N-
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27 (2,6-dimethylphenyl carbamoylmethyl) triethylammonium bromide; 10mM; Tocris) into the internal
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29 solution to block post synaptic Na⁺ currents.

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31 For Ca²⁺ imaging experiments the extracellular solution contained (in mM): 130 NaCl, 4 KCl, 2
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33 CaCl₂, 10 Glucose, 10 Hepes, 0.8 MgCl₂, 0.4 Glicine (pH 7.4).

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35 Where indicated, 6,7-Dinitroquinoxalone-2,3-Dione (DNQX, 20μM, Sigma Aldrich) and dantrolene
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37 (10μM, Sigma Aldrich) were used for blocking respectively AMPA dependent synaptic currents and
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39 RyRs.

40 41 **Results**

42 43 *Role of somatic action potential and neurotransmitter release in governing spontaneous firing of* 44 45 *hippocampal network*

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47 As already observed (Bacci A et al. 1999; Arnold FJ et al. 2004; Gavello D et al. 2012) after 18
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49 days in vitro (DIV), networks of cultured hippocampal neurons fire spontaneously (Fig. 1 a). TTX-
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51 sensitive Na⁺ channels are critical for somatic action potential (AP) generation and their block by
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53 0.3 μM TTX caused the complete inhibition of spontaneous firing (Fig.1 a, top). Glutamatergic
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55 synapses are crucial as well for governing spontaneous depolarizations (Arnold FJ et al. 2004). In
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57 particular the block of glutamate release through the administration of the selective P/Q Ca²⁺
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3 channels blocker ω -Agatoxin IV A (Fig. 1 a, middle) completely inhibited spontaneous firing by
4 preventing Ca^{2+} dependent neurotransmitter release. The selective blocker of glutamatergic AMPA
5 receptors DNQX (20 μM) completely abolished hippocampal electrical activity as well (Fig. 1 a,
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7 bottom). As previously observed (Arnold FJ et al. 2004), one hour after the block of GABA_A
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9 receptors with picrotoxin (100 μM), the firing frequency of control neurons increased from 0.9 ± 0.1
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11 Hz to 2.5 ± 0.2 Hz ($n=9$, *** $p < 0.001$) and tend to decrease over time (Fig. 1 b, c). We focused on
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13 the effect of picrotoxin after 48 to 72h from its incubation observing the generation of more regular
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15 and synchronized bursts of APs (Fig. 1 c white vs grey panels). All together these results indicate
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17 that spontaneous network depolarizations depend on both somatic APs and neurotransmitter
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19 release that mainly involves postsynaptic AMPA and GABAergic receptors.
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22 *Abeta42 oligomers inhibit spontaneous firing of hippocampal network*

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24 Although it is already known that amyloid beta peptides impair glutamatergic synaptic functions
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26 (Palop JJ and L Mucke 2010), here we focused on the effects of Abeta42 on spontaneous firing
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28 mediated by the activation of excitatory synapses. We therefore incubated neurons with Abeta42
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30 (1 μM) together with picrotoxin (Fig. 1 d) and measured the firing activity after one to 72 hours.
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32 Similarly to what observed in control neurons, after one hour of incubation with Abeta42 together
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34 with picrotoxin, firing frequency increased from 1.2 ± 0.1 Hz to 2.4 ± 0.1 Hz ($n=11$, *** $p < 0.001$) (Fig.
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36 1 b), but after 48 and 72 h (Fig. 1 d, grey panels) it was significantly reduced by Abeta42 if
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38 compared to what observed in control neurons (* $p < 0.05$). In particular, after 48h of incubation with
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40 Abeta42 and picrotoxin the firing frequency was on average 1.3 ± 0.1 Hz ($n=11$) while control
41
42 neurons displayed an average firing frequency of 1.6 ± 0.1 Hz ($n=9$). Further analysis revealed that
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44 after 48 hours of incubation with Abeta42 the intraburst firing frequency remained unaffected (Fig. 1
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46 e), while the number of bursts (Fig. 1 f), bursts duration (Fig. 1 g) and the cross correlation values
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48 (Fig. 1 h) decreased significantly. All together these results suggest that Abeta42 decreases the
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50 firing activity in mature hippocampal network. We tested the possibility that the effect of Abeta42
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52 oligomers depended on their concentration (Puzzo D et al. 2009; Lee L et al. 2013). We therefore
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54 reduced the concentration of Abeta42 to 200 nM and after 48 h of incubation we observed again a
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56 firing frequency inhibition from 1.6 ± 0.1 Hz ($n= 6$) to 1.0 ± 0.1 Hz ($n= 9$, *** $p < 0.001$, not shown). In
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3 order to understand if the effect of Abeta42 depended by its aggregation state (Lambert MP et al.
4 1998; Dahlgren KN et al. 2002), we further treated hippocampal neurons with Abeta42 previously
5 stored for 48 h at 37°C (Dahlgren KN et al. 2002). Following this procedure we observed, through
6 AFM, that Abeta42 (1 μ M) assembled in fibrils of 1-2 μ M length 5-14 nM thickness (see
7 supplementary Fig. S1). MEA recordings showed that incubation with Abeta42 fibrils for 48 h did
8 not inhibit firing frequency that was 2.2 ± 0.4 Hz (n= 5) in control and 2.6 ± 0.2 Hz (n= 4) in neurons
9 incubated with Abeta42 fibrils, $p= 0.4$) (not shown). We thus confirmed that Abeta42 dependent
10 firing inhibition is influenced by its aggregation state and is detectable during the early phase of its
11 production, when Abeta42 form oligomers rather than fibrils (Palop JJ and L Mucke 2010). From
12 here on we thus performed experiments using Abeta42 only in the oligomeric form as described in
13 the methods section at a concentration of 1 μ M. Under these conditions AFM microscopy revealed
14 that Abeta42 oligomers were characterized by a globular shape of average diameter of 10-100 nm
15 and height comprised between 2-4 nm (supplementary Fig.S1), in line with what recently proposed
16 by others (Manassero G et al. 2016).

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31 *Abeta42 oligomers increase the amplitude of spontaneous Ca^{2+} transients by potentiating*
32 *Ca^{2+} released from RyRs*

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35 Spontaneous intracellular Ca^{2+} oscillations occur regularly in primary cultured hippocampal
36 neurons (Fig.2 a). Abeta42 increased their amplitude by 46%, without affecting their frequency and
37 the basal intracellular Ca^{2+} concentration (Fig. 2 b). Average $\Delta F/F$ values increased from 1.26 ± 0.11
38 (n= 39) in control neurons to 1.84 ± 0.23 (n= 30) in neurons incubated with Abeta42 for 48 h (Fig. 2
39 c, * $p<0.05$). Being postulated the ability of Abeta42 to form Ca^{2+} permeable pores in the plasma
40 membrane (Demuro A et al. 2011), we measured whether changes in membrane resistance (R_m)
41 were detectable after incubation of neurons with Abeta42. To this purpose, we performed patch-
42 clamp recordings by holding neurons at -70-mV and injecting 50 pA negative current (not shown).
43 The degree of hyperpolarization was measured and the correspondent value of R_m calculated in
44 control neurons (0.19 ± 0.03 G Ω , n=16) was comparable with that obtained in the presence of
45 Abeta42 (0.18 ± 0.04 , n=7). The Ca^{2+} transients are generated by Ca^{2+} influx through plasma
46 membrane and Ca^{2+} released from intracellular stores when IP_3 and/or RyRs are activated.
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3 Considering the relevance of RyRs in AD onset and progression (Oules B et al. 2012; Chakroborty
4 S et al. 2013), we analyzed their contribution in the generation of spontaneous Ca^{2+} transients. As
5 already observed in chick motoneurons (Wang S et al. 2009) here we found that in control neurons
6 RyRs contribute to the generation of spontaneous Ca^{2+} transients (Fig.2 d), but they are scarcely
7 involved in the regulation of firing activity (Cheong E et al. 2011) (Fig. 2 e). The selective RyRs
8 negative allosteric modulator dantrolene (10 μM) reduced indeed the peak amplitude of
9 spontaneous Ca^{2+} transients by $31.9 \pm 8.7\%$ ($n=8$) (Fig. 2 f) without altering the firing frequency (Fig.
10 2 g) and increasing by $17.0 \pm 7.7\%$ the cross correlation (Fig. 2 h). Dantrolene mediated inhibition of
11 Ca^{2+} transients in neurons treated with Abeta42 for 48 h (Fig. 2 i, f) was significantly larger than in
12 control conditions ($54.0 \pm 5.6\%$, $n=8$, $*p<0.05$), while the firing frequency (Fig. 2 l, g) and the cross
13 correlation (Fig. 2 h) values increased respectively by $13.3 \pm 5.5\%$ ($n=9$) and $53.6 \pm 14.8\%$ (**
14 $p<0.001$) after 48 h of incubation with Abeta42. To finally test if RyRs are targeted by Abeta42, we
15 measured the amount of Ca^{2+} released through RyRs by stimulating neurons with the RYRs
16 agonist caffeine (10 mM) (see supplementary Fig. S2). We found that the average $\Delta F/F$ value was
17 significantly ($* p<0.05$) higher in Abeta42 treated neurons (0.9 ± 0.1 , $n=6$) with respect to control
18 (0.3 ± 0.1 , $n=6$). We then concluded that Abeta42 increases the amount of Ca^{2+} released through
19 RyRs and that its inhibition counteracts the effects of Abeta42 on spontaneous Ca^{2+} transients,
20 firing frequency and synchronization of neuronal hippocampal network.

21 *Abeta42 oligomers inhibit spontaneous firing through BK channels activation*

22 We hypothesized that Ca^{2+} activated potassium channels (IK_{Ca}) (Marrion NV and SJ Tavalin 1998)
23 could be responsible for Abeta42 dependent inhibition of neuronal firing following the increased
24 amplitude of spontaneous Ca^{2+} transients. We first focused on small conductance potassium
25 channels (SK channels) (see supplementary Fig. S3) (Stocker M et al. 1999; Cheong E et al. 2011)
26 observing that in control conditions the administration of the SK channels blocker apamin (200 nM)
27 did not significantly change the firing frequency (1.5 ± 0.1 Hz ($n=6$) in control vs 1.4 ± 0.1 Hz ($n=6$)
28 with apamin ($p=0.16$)), while the cross correlation value increased by $20.0 \pm 5.0\%$. We then
29 concluded that in control conditions, SK channels are mainly involved in controlling network
30 synchronization. When SK channels were blocked in neurons previously incubated with Abeta42
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3 (supplementary Fig. S3), we observed that the overall firing frequency decreased from 1.2 ± 0.2 Hz
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5 ($n=6$) to 1.0 ± 0.2 Hz, ($***p < 0.001$) while the cross correlation value was unaffected or slightly
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7 inhibited ($-8.0 \pm 3.0\%$). We concluded that SK channels inhibition does not contribute to recover the
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9 physiological firing parameters impaired by Abeta42.

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11 Big conductance (BK) channels are involved in controlling firing rate (Storm JF 1987) and synaptic
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13 function (Raffaelli G et al. 2004) of rat hippocampal neurons. They were blocked by paxilline (1
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15 μM) (Marcantoni A et al. 2010; Zhou Y and CJ Lingle 2014), but the firing frequency of control
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17 neurons was unaffected or slightly inhibited ($-8.8 \pm 4.3\%$, $n=5$) (Fig.3 a) and the cross correlation
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19 value remained unaltered (not shown). These results suggest that in control conditions BK
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21 channels are not primarily involved in governing spontaneous firing of hippocampal network. On
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23 the contrary, when neurons were incubated for 48 h with Abeta42 (Fig. 3 b), paxilline potentiated
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25 the firing frequency by $32.1 \pm 5.6\%$ ($n= 6$) (Fig. 3 c) without altering the cross correlation value (not
26
27 shown). This result suggests that BK channels function is stimulated by Abeta42 and that they
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29 contribute to inhibit the spontaneous firing observed in hippocampal network. To test this
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31 hypothesis we performed patch clamp experiments in voltage clamp configuration (Fig. 3 d, e). The
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33 maximum amplitude of K^+ current measured in voltage clamp experiments by depolarizing neurons
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35 from -70 to $+80$ mV for 400 ms was comparable among control and Abeta42 treated neurons and
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37 equal to 3.5 ± 0.4 nA ($n= 12$) in control versus 3.3 ± 0.5 nA ($n= 10$) in the presence of Abeta42.
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39 Though, the effect of paxilline was significantly potentiated by Abeta42. In particular, $14.7 \pm 2.3\%$
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41 ($n= 12$) of the total outward potassium current is carried by BK channels in control neurons (Fig. 3
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43 f), while in neurons treated with Abeta42 the BK current contribution is significantly ($* p < 0.05$)
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45 increased to $23.4 \pm 3.2\%$ ($n= 10$). When the depolarizing step was preceded by stimuli of increased
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47 duration (from 10 to 90 ms) to -10 mV to open all available voltage-gated Ca^{2+} channels
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49 (Marcantoni A et al. 2010) we did not measure any further increase of the outward current (see
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51 supplementary Fig S4) suggesting that BK channels are maximally activated even in the absence
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53 of the above mentioned pre-step protocol. All together these results suggest that Abeta42
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55 stimulates the paxilline sensitive outward current carried by BK channels.
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58 *Presynaptic BK channels are upregulated by Abeta42*
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3 It is generally accepted that, in neurons, BK channels are distributed both at somatic and synaptic
4 sites (Raffaelli G et al. 2004; Martire M et al. 2010). In order to understand if somatic BK channels
5 are targeted by Abeta42, we tested if the increased intracellular Ca^{2+} concentration induced by
6 Abeta42 could affect AP duration (Marcantoni A et al. 2014). We therefore performed whole cell
7 current clamp recordings and measured the width of the first AP generated by injecting squared
8 pulses of depolarizing currents (see Methods). In control neurons (Fig.3 g) paxilline widened APs
9 and the half width increased from 1.8 ± 0.3 ms to 2.0 ± 0.3 ms ($n=17$, * $p<0.05$) similarly to what
10 observed in neurons treated with Abeta42 (Fig.3 h) where the half width value increased from
11 1.6 ± 0.2 ms to 1.9 ± 0.3 ms ($n=24$, * $p<0.05$). We therefore concluded that somatic BK channels are
12 not affected by Abeta42 and focused on synaptic BK channels. We first measured the
13 spontaneous eEPSCs following AP generation (see Methods). Administration of DNQX (20 μ M)
14 together with picrotoxin completely blocked eEPSCs (not shown) thus suggesting that they are
15 mainly mediated by AMPA receptor activation. In control neurons (Fig. 3 i), neither the amplitude
16 (Fig. 3 l), nor the frequency (Fig. 3 m) of AMPA dependent eEPSCs were affected by paxilline. The
17 average amplitude was respectively -12.8 ± 0.2 pA in control and -12.4 ± 0.2 pA with paxilline ($n= 12$,
18 $p= 0.14$), while the inter event interval (IEI) was 147.6 ± 3.3 ms and 155.7 ± 4.5 ms with paxilline ($p=$
19 0.13). We then concluded that in physiological conditions, while BK channels contribute to AP
20 duration, they are not involved in controlling the AMPA dependent glutamatergic synapses. In good
21 agreement with the decreased firing activity induced by Abeta42 administration (Fig. 1 b-d), when
22 we compared control neurons with those treated with Abeta42 (Fig. 3 n), the eEPSCs amplitude of
23 these latter increased significantly to -21.7 ± 0.6 pA ($n= 10$, Fig.3 o) and was not sensitive to the
24 presence of paxilline (-21.1 ± 0.6 pA, $p= 0.48$). IEI values were significantly higher as well
25 (189.3 ± 7.7 ms, Fig. 3 p) and with paxilline they decreased to 131.3 ± 5.9 ms (** $p<0.001$). All
26 together these results suggest that, being Abeta42 able to alter both IEI and amplitude of eEPSCs,
27 it targets AMPA dependent glutamatergic synapses at pre and post synaptic sites. Being only the
28 IEI values affected by the presence of paxilline, we concluded that presynaptic BK channels
29 dependent current is stimulated by Abeta42.
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3 Although in control neurons BK channels are not involved in controlling spontaneous firing (Fig. 3
4 a), we tested the hypothesis that these latter could be recruited when the amount of Ca^{2+} released
5 by RyRs increased, thus determining glutamatergic AMPA dependent current inhibition. To this
6 purpose we measured electrically evoked monosynaptic AMPA dependent glutamatergic EPSCs
7 (eEPSCs) in control conditions and after administration of caffeine and paxilline. These two
8 compounds were used for respectively stimulate Ca^{2+} release from RyRs and inhibit BK channels.
9 We observed that activation of RyRs by caffeine inhibited eEPSCs by 64% (from 152.0 ± 73.0 pA,
10 $n = 28$ to 54.2 ± 23.9 pA, $n = 8$, * $p < 0.05$ by ANOVA) (Fig. 4 a, d). Because this effect was abolished
11 by paxilline, we concluded that, when Ca^{2+} released by RyRs increases, it activates BK channels
12 which in turn inhibit AMPA dependent eEPSCs. We next tested whether in control neurons the
13 inhibition of RyRs and/or BK channels could affect AMPA dependent synaptic transmission.
14 Monosynaptic eEPSCs were first measured in the presence of paxilline (Fig. 4 b, d) and their
15 average amplitude (142.0 ± 19.3 pA, $n = 20$) was comparable to control conditions, unlike to what
16 observed in Abeta42 treated neurons (Fig. 4 c, d), where the average eEPSCs amplitude
17 (177.8 ± 18.6 pA, $n = 28$) was increased by paxilline to 291.7 ± 70.6 pA ($n = 10$, * $p < 0.05$). Dantrolene
18 (Fig. 4 b, e) did not change the average eEPSCs amplitude measured in control neurons
19 (145.6 ± 21.0 , $n = 15$), that instead increased to 368.3 ± 70.4 pA ($n = 11$, * $p < 0.05$) after incubation
20 with Abeta42 (Fig. 4 c, e). Simultaneous administration of dantrolene together with paxilline did not
21 alter significantly the amplitude of eEPSCs in control neurons (168.0 ± 42.0 pA, $n = 15$, Fig. 4 b, e),
22 but abolished the potentiating effect observed in Abeta42 treated neurons, making the average
23 eEPSCs amplitude (141.2 ± 37.2 pA, $n = 6$) comparable to that measured before RyRs and BK
24 channels inhibition (Fig. 4 c, e). These results confirm that Abeta42 inhibits the amplitude of AMPA
25 dependent eEPSCs by stimulating RyRs and BK channels function.

26 *NMDA receptors are inhibited by Abeta42 oligomers*

27 DNQX (20 μM) and TTX (0.3 μM) completely abolished spontaneous Ca^{2+} transients (Fig. 5 a),
28 suggesting that AMPA receptors (AMPA) and voltage gated Na^+ channels control Ca^{2+}
29 oscillations by governing respectively synaptic and somatic dependent depolarizations.

30 Alternatively to AMPARs, NMDA receptors (NMDARs) as well can be activated by glutamate

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3 released at excitatory synapses and mediate Ca^{2+} entering into neurons. We therefore focused on
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5 the mechanisms of Ca^{2+} entry through NMDARs by measuring their contribution in the regulation of
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7 Ca^{2+} influx both in control (Fig. 5 a) and in the presence of Abeta42 (Fig.5 b). The selective
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9 NMDARs blocker APV (50 μM) inhibited Ca^{2+} transients amplitude in control neurons by 68.5 ± 7.2
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11 % and this effect was more pronounced ($***p < 0.001$) than in Abeta42 treated neurons (47.7 ± 4.2 %
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13 of inhibition) (Fig.5 c). We next tested the effect of APV on spontaneous firing observing that it was
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15 inhibited by 59.8 ± 2.6 % (n= 5) in control neurons (Fig. 5 d, f). This effect was significantly
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17 ($***p < 0.001$) reduced to 33.6 ± 7.3 % (n= 4) in Abeta42 treated neurons (Fig. 5 e, f). Cross
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19 correlation analysis (Fig. 5 g) showed that APV increased network synchronization by 25.9 ± 5.5 %
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21 in control neurons and that this effect was significantly ($*** p < 0.001$) more pronounced in the
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23 presence of Abeta42 (89.7 ± 12.8 %). All together these results suggest that Abeta42 inhibits
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25 NMDARs and that these latter are important for governing both spontaneous network
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27 depolarizations and Ca^{2+} oscillations.

28 29 *Abeta42 inhibits Ca^{2+} entry through VGCCs*

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31 Together with NMDARs, voltage gated Ca^{2+} channels (VGCCs) regulate Ca^{2+} entry through plasma
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33 membrane (Alford S et al. 1993; Goussakov I et al. 2010). We found that when cadmium (200 μM)
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35 was added to extracellular medium together with APV, it completely abolished intracellular Ca^{2+}
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37 oscillations (Fig.6 a), suggesting that, in control neurons, VGCCs together with NMDARs are
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39 important for governing spontaneous Ca^{2+} transients. We therefore performed patch clamp
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41 experiments to verify whether Abeta42 affect VGCCs, by considering both L- and non-L-type
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43 channels known to be largely expressed in hippocampal neurons (Baldelli P et al. 2000; Baldelli P
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45 et al. 2002). In control neurons (Fig.6 b) after 11-14 DIV the total Ca^{2+} current at -10 mV in 2 mM
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47 Ca^{2+} was -692.8 ± 80.5 pA (n= 24) (Fig. 6 c). Addition of 3 μM nifedipine reduced the peak current
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49 amplitude to -430.3 ± 55.6 pA (Fig. 6 b, c) indicating that L-type Ca^{2+} channels (LTCCs) contribute to
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51 38 % of total Ca^{2+} current (256.1 ± 37.4 pA), while the remaining 62 % of total Ca^{2+} current is carried
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53 by non-LTCCs. In the presence of Abeta42 the amount of total Ca^{2+} current (Fig.6 d) significantly
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55 decreased to -284.5 ± 40.0 pA (n=17, $***p < 0.001$). When LTCCs were blocked with nifedipine, the
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57 remaining Ca^{2+} current was -162.6 ± 31.8 pA (Fig. 6 c, d). The contribution of LTCCs was slightly
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3 but not significantly increased to 42.6 % and the remaining 57.4 % of the total Ca^{2+} current carried
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5 by non-LTCCs was comparable to that of control neurons. We then concluded that Abeta42
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7 oligomers decreased the amplitude of Ca^{2+} current carried by VGCCs without altering the
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9 proportion between L and non-LTCCs.

10 11 **Discussion**

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13 We studied the effects of APP derived protein Abeta42 on neuronal network excitability. Our
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15 results suggest that, after 48h of incubation, Abeta42 inhibits spontaneous firing and increases
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17 Ca^{2+} transients amplitude. We suggest that Abeta42 is responsible for remodeling intracellular Ca^{2+}
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19 homeostasis as a consequence of RyRs stimulation followed by NMDARs and VGCCs inhibition.
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21 All together these effects contribute to reduce the network excitability through BK channels
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23 activation and inhibition of AMPA dependent synaptic current. Physiological excitatory parameters
24
25 are partially restored through the inhibition of RyRs or BK channels suggesting that their selective
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27 target could be useful for treating early symptoms of AD.

28 29 *Inhibition of spontaneous firing by Abeta42*

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31 Our previous reports (Gavello D *et al.* 2012; Allio A *et al.* 2015) show that primary cultures of
32
33 hippocampal neurons generate spontaneous bursts of APs recorded extracellularly by means of
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35 MEAs. Here we show that spontaneous firing depends on both somatic and synaptic excitability
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37 (Fig. 1 a). In particular, we clearly define the degree of importance of both glutamatergic and
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39 GABAergic synapses in governing network excitability and show that, while it is completely
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41 abolished by blocking glutamatergic AMPA receptors, it is only partially altered when GABAergic
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43 (Fig. 1 c) and NMDARs (Fig. 5 d) are inhibited. Here we focused on the role of glutamatergic
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45 AMPA-dependent synapses to regulate spontaneous firing. However, future experiments will be
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47 needed for clarify any specific effect induced by Abeta42 on the GABAergic counterpart.

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49 It is already well established that in AD the mechanisms of synaptic transmission are impaired (Ye
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51 H *et al.* 2010; Chong SA *et al.* 2011), but recent reports suggest that intrinsic neuronal excitable
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53 properties are modified too (Marcantoni A *et al.* 2014; Tamagnini F *et al.* 2015). These effects are
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55 often contradictory and not well understood, probably because many causal agents that contribute
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57 to AD onset exist and many animal models have been proposed to be suitable for studying this
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3 pathology. A great quantity of data comes from experiments on AD transgenic animals that exhibit
4 Abeta accumulation (Marchetti C and H Marie 2011). Among various forms of Abeta oligomers,
5 Abeta42 is considered particularly toxic for neurons (Lambert MP et al. 1998). Here we focus on its
6 effects on both spontaneous network excitability and Ca^{2+} oscillations.
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10 One of the major issues concerning the use of Abeta42 is represented by its tendency to
11 aggregate during time (Dahlgren KN et al. 2002; Puzzo D et al. 2009) and to exert different effects
12 depending on its concentration that in turn affects the aggregation process as well (Mucke L and
13 DJ Selkoe 2012). We therefore identified the main structure and concentration of Abeta42
14 responsible for neuronal dysfunction. We suggest that Abeta42 oligomers, rather than fibrils, are
15 the main responsible for neuronal dysfunction. This conclusion agrees with most of the data
16 presented in literature, but contrasts with others (Minkeviciene R et al. 2009) which identify
17 Abeta42 fibrils as the main cause of the increased neuronal excitability observed. Being our
18 experimental conditions significantly different from those reported by Minkeviciene et al. (e.g.
19 Abeta42 concentration and time of exposure), we concluded that these conflicting results cannot
20 be compared and we do not exclude that high concentrations (100 μ M) of fibrils of Abeta42 during
21 shorter period of incubations (1 h) could increase neuronal excitability. Moreover, the Abeta42
22 dependent effects here described could be presumably influenced by the presence of other Abeta
23 peptides such as Abeta40. These latter may in turn influence the Abeta aggregation process as
24 already reported (Kuperstein I et al. 2010). Future experiments will be necessary to clarify this
25 issue. Finally, new insights about the effects of Abeta42 are provided in this work by MEA
26 recordings: we demonstrate that the spontaneous neuronal firing is inhibited (Fig. 1 b, d), as well
27 as the number and the duration of bursts (Fig. 1 f, g). Burst activity is critical for neuronal function
28 since during those prolonged excitatory periods, typical of mature networks, Ca^{2+} enters into
29 neurons and regulates many neuronal activities as well as neurotransmitter release. Furthermore,
30 we clearly show that Abeta42 inhibits network synchronization (Fig. 1 h), causing, in this way, the
31 reduction of effectiveness of synaptic input (Marcantoni A et al. 2014).
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55 *ABeta42 oligomers stimulate spontaneous calcium transients by mainly targeting RYRs.*
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3 As already observed in different neurons (Murphy TH et al. 1992; van den Pol AN et al. 1992;
4 Bacci A et al. 1999; Wang S et al. 2009) network of primary cultured hippocampal neurons
5 generate spontaneous Ca^{2+} transients (Fig. 2 a) of longer duration than bursts of APs. Here we
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7 show that bursts of APs occur on average 10 folds faster than Ca^{2+} transients (hundred ms
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9 duration vs s), suggesting that several bursts of APs occur during one single Ca^{2+} transient. We
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11 also suggest that a correlation between Ca^{2+} transient amplitudes and firing frequency exists and
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13 the general rule is that by increasing the amplitude of Ca^{2+} transients the firing frequency is
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15 inhibited. Several studies demonstrate that Ca^{2+} dyshomeostasis is early observed in AD and it can
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17 contribute to generate synaptic dysfunction. We aimed at evaluating the effect of ABeta42 on
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19 spontaneous Ca^{2+} transients and how these could be correlated with altered neuronal network
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21 excitability. To this regard, it has been proposed that Abeta42 could create pores into the plasma
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23 membrane and that these latter could contribute to determine Ca^{2+} dyshomeostasis phenomena
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25 (Demuro A et al. 2011). Our experiments show that neither the basal intracellular Ca^{2+}
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27 concentration (Fig. 2 b), nor the R_m value are altered by Abeta42. Thus we can conclude that, in
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29 our experimental conditions, Abeta42 does not form Ca^{2+} permeable pores. Increasing evidences
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31 support the idea that RyRs activity is upregulated in AD, but most of the studies are limited to
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33 transgenic mice (Oules B et al. 2012; Chakroborty S et al. 2013) and only few reports propose an
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35 effect of Abeta42 on RyRs (Supnet C et al. 2006; Lazzari C et al. 2014). Here, by blocking RyRs
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37 with dantrolene (Fig. 2) and stimulating them with caffeine (supplementary Fig. S2) we clearly
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39 show their increased function induced by Abeta42. As already observed in thalamocortical neurons
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41 (Cheong E et al. 2011), we propose that under basal physiological conditions the spontaneous
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43 firing network is not affected by Ca^{2+} released through RyRs. On the other hand it is known that
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45 when the duration (Cheong E et al. 2011) or the frequency (Riquelme D et al. 2010) of electrical
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47 stimuli increases, the amount of Ca^{2+} released by RyRs increases as well and becomes involved in
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49 setting the neuronal firing frequency. Similarly to what above described, here we suggest that
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51 when ABeta42 targets RyRs the amount of intracellular Ca^{2+} concentration increases enough to
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53 inhibit firing network. To support this hypothesis, we finally demonstrate that dantrolene is able to
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55 reverse the inhibitory effects of Abeta42 on firing activity and synchronization of hippocampal
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3 network. Currently, the precise effect of Abeta42 on RyRs is still to be elucidated. Future
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5 experiments are needed to characterize whether Abeta42 is responsible for the increased Ca^{2+}
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7 loading of intracellular stores or for the increased number and/or conductance of available RyRs.
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9 To this regard, it has been recently shown (Barucker C et al. 2014) that, already after 30 min of
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11 incubation, Abeta42 is found in the nucleus and interferes with gene transcription. We therefore
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13 cannot exclude the hypothesis that after 48-72 h of incubation Abeta42 could interfere with RyRs
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15 gene transcription process. It is worth noticing that here we do not consider the effect of Abeta42
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17 on IP_3 receptors. Many reports suggest that during AD the amount of Ca^{2+} released through IP_3
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19 receptors is increased as well (Stutzmann GE et al. 2004; Oules B et al. 2012; Chakroborty S et al.
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21 2013), but other recent reports indicate that their function is inhibited by Abeta42 (Lazzari C et al.
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23 2014). Despite these contrasting results, it is well accepted that Ca^{2+} released through IP_3
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25 receptors concurs to the activation of RyRs and that dantrolene treatment suppresses Ca^{2+} release
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27 from both receptors (Chakroborty S et al. 2013) thus confirming the effectiveness of dantrolene
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29 during AD onset.

31 *BK channels are targeted by Abeta42*

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33 Our data demonstrate that Abeta42, besides inhibiting neuronal firing and synchronization,
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35 increases the amplitude of intracellular Ca^{2+} oscillations, thus suggesting a possible involvement of
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37 Ca^{2+} activated potassium channels (IK_{Ca}). Two classes of IK_{Ca} with different affinity for Ca^{2+} exist.
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39 They are represented by Ca^{2+} dependent small conductance (SK) and voltage and Ca^{2+} dependent
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41 large conductance (BK) K^+ channels (Fakler B and JP Adelman 2008). The former govern firing
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43 rate, adaptation, regularity of APs firing. Their block prevents the repolarization at the end of the
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45 single AP, thus decreasing the number of Na^+ channels available for the next spike, increasing AP
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47 width and bringing neuron to a depolarizing block condition (Vandael DH et al. 2015). This
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49 mechanism could explain the inhibition of firing frequency and synchronism induced by apamin on
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51 spontaneous firing of hippocampal neurons treated with Abeta42 (Fig. S3), similarly to what
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53 observed in AD mice models where the synaptic function is altered by upregulation of SK channels
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55 (Chakroborty S et al. 2012). However, since SK channels inhibition does not contribute to recover
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57 the physiological firing parameters impaired by Abeta42, therefore it is reasonable to assume they
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3 do not represent a promising target for restoring impaired neuronal function. We next focused on
4 the role of BK channels, characterized by lower degree of affinity for Ca^{2+} with respect to SK, being
5 activated by higher intracellular Ca^{2+} concentration (more than $10 \mu\text{M}$) (Vandael DH et al. 2015).
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7 For these reasons, in neurons or neuronal like cells, BK channels are co-localized with the Ca^{2+}
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9 source mainly represented either by Ca^{2+} channels (Marrion NV and SJ Tavalin 1998; Marcantoni
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11 A et al. 2010; Vandael DH et al. 2010), or by RyRs as well (Chavis P et al. 1998; Beurg M et al.
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13 2005). It is generally accepted that BK channels are located at both somatic and synaptic sites (Hu
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15 H et al. 2001) and are respectively important for controlling the AP shape (Storm JF 1987) and the
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17 neurotransmitter release (Raffaelli G et al. 2004; Martire M et al. 2010). Similarly to what already
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19 described (Hu H et al. 2001), here we show that in control neurons BK channels contribute little to
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21 the total K^+ activated current (Fig. 3 d) even when increased amount of Ca^{2+} enters into neurons
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23 (supplementary Fig. S4). We therefore conclude that they regulate the AP shape (Fig. 3 g) but are
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25 scarcely involved in controlling spontaneous firing of hippocampal network (Fig. 3 a) and do not
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27 regulate neurotransmitter release (Fig. 3 i, 4 b), unless RyRs are strongly activated (Fig. 4 a). On
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29 the contrary, in the presence of Abeta42, the increased amount of intracellular Ca^{2+} concentration
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31 potentiates BK channels activation (Fig. 3 e, f) and this effect is specific for the presynaptic (Fig. 3
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33 n, 4 c), but not somatic (Fig. 3 h) compartment. We in fact show that paxilline increases the
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35 frequency (Fig. 3 p), but not the amplitude of spontaneous eEPSCs following AP generation (Fig. 3
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37 o) and increases the amplitude of electrically evoked eEPSCs (Fig. 4 e), while the AP duration is
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39 comparable to that measured in control neurons (Fig. 3 g, h). Further experiments will be
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41 necessary to understand the molecular mechanism responsible for BK channels activation induced
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43 by Abeta42 since we cannot exclude that the density and/or the conductance of these channels
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45 are increased. To this regard, a recent report (Donnelier J et al. 2015) suggests that Abeta42 does
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47 not increase the expression of the BK α subunit expression reinforcing the hypothesis of increased
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49 BK channels conductance. Finally, the experiments here discussed do not only demonstrate the
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51 activation of BK current by Abeta42, in good agreement with previous findings (Ye H et al. 2010).
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53 They also indicate the presence of a strong link between the increased amount of Ca^{2+} released by
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55 RyRs, the activation of BK channels and inhibition of the amplitude of glutamatergic AMPA
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3 dependent current (Fig. 4 a). These overall effects could be responsible for decreasing the rate of
4 glutamate release and in turn the rate of spontaneous firing of hippocampal network. It is
5 noteworthy that while the frequency of eEPSCs in neurons treated with ABeta42 is significantly
6 reduced when compared to control conditions, their amplitude is higher (Fig.3 l, o). We therefore
7 conclude that presynaptic BK channels could be preferentially coupled with RyRs and that
8 postsynaptic AMPA receptors function is upregulated by ABeta42, but the specific effects induced
9 by Abea42 respectively at pre and post synaptic glutamatergic compartment are still to be
10 investigated, as well as the molecular pathway that determines the increased amount of Ca^{2+}
11 released by RyRs. Finally, we cannot also exclude that a coupling between IP_3 receptors and BK
12 channels could exist, but, while these latter are mainly distributed at somatic region, RyRs prefer
13 synaptic sites (Goussakov I et al. 2010; Chakroborty S et al. 2012) where BK channels are
14 upregulated. All together these results strongly indicate BK channels as a target for treating early
15 symptoms of AD.

29 *NMDARs and VGCCs are inhibited by Abeta42*

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31 NMDARs are important for controlling spontaneous Ca^{2+} transients and it is well accepted that they
32 are inhibited during aging (Thibault O et al. 2007) and in AD (Yu JT et al. 2009). This mechanism
33 of Ca^{2+} influx is thought to be involved in governing neuronal plasticity, learning and memory
34 processes (Malenka RC et al. 1988) that are known to be impaired in AD (LaFerla FM 2002). Our
35 data add new insights about the effect of ABeta42 in the regulation of intracellular Ca^{2+}
36 concentration by NMDARs. First we demonstrate that Ca^{2+} transients recorded in cultured
37 hippocampal neurons depend by both somatic APs generation and activation of glutamatergic
38 synapses (Fig. 5 a). We in fact show that TTX administration abolishes completely Ca^{2+} oscillations
39 and the same is observed by blocking AMPA receptors through DNQX. Our data suggest that
40 AMPA receptors are essential for spontaneous generation of Ca^{2+} transients while NMDA
41 contribute only partially to this phenomenon and confirm what already observed in rat derived
42 hippocampal neurons (Bacci A et al. 1999). The involvement of NMDA receptors in governing
43 spontaneous firing of hippocampal network (Fig. 5 d) has been studied too. Our results show that
44 their inhibition contributes only in part to the recovery of neuronal firing properties impaired by
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3 Abeta42, by increasing network synchronization, but not firing frequency, thus suggesting that the
4 effectiveness of therapies for AD treatment based on NMDA receptors blockers actually employed
5 mainly depends on their ability to preserve the neuronal network synchronism. We finally observed
6 that the effect of APV on Ca^{2+} transients and firing is less pronounced in the presence of Abeta42
7 (Fig. 5 b, c). Even if future experiments will be needed for clarifying this issue, all together these
8 results suggest that NMDARs and the spontaneous firing activity of hippocampal networks are
9 inhibited by Abeta42.
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18 Hippocampal pyramidal neurons express high densities of L-type Ca^{2+} channels (LTCCs) in the cell
19 body and proximal dendrites. They contribute to increase Ca^{2+} entry following NMDARs activation
20 and together with NMDARs are involved in the control of Ca^{2+} influx and neuronal plasticity in
21 hippocampus (Westenbroek RE et al. 1990). In addition, VGCCs are important to regulate the
22 excitability of neurons (Bean BP 2007) or neuronal like cells (Marcantoni A et al. 2009; Marcantoni
23 A et al. 2010; Vandael DH et al. 2015) Several reports indicate that their contribution to Ca^{2+} entry
24 through plasma membrane increases during age when they are up-regulated (Thibault O et al.
25 2007; Yu JT et al. 2009). LTCCs upregulation has been frequently observed during AD (Wang Y
26 and MP Mattson 2013), but opposing evidences suggest that they can be inhibited as well
27 (Thibault O et al. 2012). This underlies the variability of effects on Ca^{2+} homeostasis observed in
28 AD probably correlated with the multifactorial causes of the pathology together with different
29 responses of the brain region of interest. Here we show their involvement in the spontaneous
30 generation of Ca^{2+} transients (Fig. 6 a) and the inhibition of both L and non-LTCCs isoforms (Fig. 6
31 b - d) induced by Abeta42. In conclusion, the present work clarifies the effects of Abeta42 on
32 neuronal function and, by focusing on the mechanisms that regulate Ca^{2+} oscillations, firing,
33 synaptic function, correlates data from neuronal networks with those on isolated neurons. We
34 suggest that Abeta42 increases the amplitude of Ca^{2+} transients, decreases the firing rate and
35 network synchronization by mainly increasing RyRs and BK channels functions and inhibiting
36 NMDARs and VGCCs (Fig. 6 e). The modulation of these targets would represent a promising
37 strategy for early treatment of AD. We wish finally to point out that previous results obtained by our
38 group (Marcantoni A et al. 2014). revealed that excitatory profile of neurons from lateral entorhinal
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3 cortex is early altered during AD onset. These results indicate that sensory inputs involved in
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5 memory formation, including olfactory information processing conveyed by lateral entorhinal cortex
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7 to hippocampus, might be early compromised and suggest how new diagnostic methods for an
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9 early identification of AD should be addressed.
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24 **Figure legends**

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26 Fig.1
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29 a) Spontaneous hippocampal electrical activity measured by means of MEAs by one
30 representative recording channel at 18 DIV after one hour of incubation with picrotoxin. *top*)
31 Spontaneous hippocampal electrical activity depends on the availability of TTX-sensitive somatic
32 Nav1 channels. *middle*) The block of glutamate release through administration of presynaptic Cav
33 2.1 channels blocker ω -Agatoxin IVA (2 μ M) completely inhibits spontaneous firing by preventing
34 Ca²⁺ dependent neurotransmitter release. *bottom*) The selective AMPA receptors inhibitor DNQX
35 completely abolishes hippocampal electrical activity **b**) Time course of hippocampal network firing
36 frequency recorded from 1 to 72 hours after incubation with picrotoxin alone (control, dark grey
37 diamonds) and together with Abeta42 (1 μ M) (light grey squares). After 48 and 72 hours of
38 incubation with picrotoxin the firing frequency is significantly decreased by Abeta42. **c**) Selection of
39 three representative recording traces from the same MEA showing neuronal spontaneous firing in
40 control conditions at 18 DIV (white) and after exposure to picrotoxin (100 μ M) for respectively 48
41 (light grey panel) and 72 h (dark grey panel). The two insets refer to the magnification of single
42 bursts of action potentials. **d**) Three representative recording traces from the same MEA showing
43 the inhibition of spontaneous firing by Abeta42 (1 μ M). Neuronal firing was recorded at 18 DIV
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3 before administration of Abeta42 and picrotoxin (white panel) and compared to that measured after
4 48h (light grey panel) and 72 h (dark grey panel) from administration of Abeta42 together with
5 picrotoxin. After 48 h (light grey bar) the intraburst firing frequency remains unaffected (**e**) while
6 both number of bursts (**f**), bursts duration (**g**) and cross correlation (**h**) decrease significantly with
7 respect to controls (dark grey bar).
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14 Fig.2

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17 **a)** After 18 DIV hippocampal network generates spontaneous Ca^{2+} transients whose amplitude is
18 significantly increased (**b**) after incubation for 48 hours with Abeta42. **c)** Bar graph summarizing
19 the increased amplitude of Ca^{2+} transients induced Abeta42 (light grey bar) with respect to controls
20 (dark grey bars). **d)** In control conditions, the inhibition of RyRs with dantrolene decreases the
21 amplitude of Ca^{2+} transients. **e)** MEA recording showing that in control neurons spontaneous firing
22 is not influenced by dantrolene. **f)** Bar graph comparing the more pronounced inhibition of Ca^{2+}
23 transients amplitude induced by dantrolene on Abeta42 treated cells (light grey bar) with respect to
24 control (dark grey bar). In neurons treated for 48h with Abeta42 the effect of dantrolene is
25 potentiated. **g)** Bar graph showing that when neurons are exposed to Abeta42 for 48 h (light grey
26 bar), dantrolene increases the firing frequency and **h)** cross correlation probability. These effects
27 are more pronounced than that observed in control neurons (dark grey bar). **i)** In neurons treated
28 for 48h with Abeta42 the effect of dantrolene on the amplitude of Ca^{2+} transients is potentiated with
29 respect to control (d). **l)** MEA recording showing that in Abeta42 treated neurons dantrolene
30 increases the frequency of spontaneous firing.
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46 Fig. 3

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49 **a)** Three representative channels from the same MEA showing that while spontaneous firing is not
50 altered by paxilline (1 μM) in control neurons, it is increased (**b**) in those treated for 48 hours with
51 Abeta42. **c)** Bar graph summarizing that firing frequency of cultured hippocampal network is
52 unaffected or slightly inhibited by paxilline in control neurons (dark grey bar), while it is increased in
53 Abeta42 treated neurons (light grey bar). **d)** Paxilline (light grey trace) inhibits the total outward K^+
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3 current (black trace) in control. **e**) The inhibition of K^+ current induced by paxilline is potentiated
4 after 48 hours of incubation with Abeta42. **f**) Bar graph summarizing the contribution of BK
5 activated current with respect to the total amount of K^+ current activated in hippocampal neurons in
6 control conditions (dark grey bar) and in presence of Abeta42 (light grey bar). **g**) Paxilline (light
7 grey trace) broadens the action potential shape in control neurons (black trace). The effect is
8 comparable to that measured in neurons incubated for 48h with Abeta42 (**h**). The dotted lines in **g**,
9 **h** correspond to membrane potential of 0 mV. **i**) Representative recording of AMPA eEPSCs
10 elicited by spontaneous APs in control conditions. Paxilline does not modify neither the amplitude
11 (**l**) nor the frequency (**m**) of eEPSCs following AP generation. **n**) In neurons treated with Abeta42
12 paxilline does not alter eEPSCs amplitude (**o**) but decreases the inter event interval (IEI) (**p**).

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27 **a**) In control neurons, electrically evoked AMPA eEPSCs amplitude is significantly inhibited by
28 caffeine (10mM) while paxilline abolishes this effect, **b**) Administration of paxilline and dantrolene
29 simultaneously or separately does not change the average amplitude of eEPSCs measured in
30 control neurons. **c**) After incubation with Abeta42 oligomers for 48h eEPSCs amplitude increases
31 when paxilline or dantrolene are administered separately. This effect is abolished when the two
32 compounds are administered together. **d**) Bar graphs comparing the average eEPSCs amplitude in
33 control neurons (dark grey bars) with that measured in neurons incubated 48h with Abeta42 (light
34 grey bars). The average eEPSCs amplitudes of the two populations are comparable and are
35 inhibited by caffeine in control neurons. This effect is abolished by paxilline. In control neurons
36 paxilline alone does not interfere with the average eEPSCs amplitude, which is increased in
37 neurons previously incubated with Abeta42. **e**) Bar graphs summarizing the effect of dantrolene
38 alone and together with paxilline in control neurons (dark grey bars) and in those incubated for 48h
39 with Abeta42 (light grey bars). eEPSCs amplitude from control neurons are not affected by
40 dantrolene administered alone or together with paxilline. Dantrolene potentiates the eEPSCs
41 amplitude in neurons previously incubated with Abeta42. This potentiating effect induced by
42 dantrolene is abolished by paxilline.

Fig.5

a) DNQX completely abolishes Ca^{2+} oscillations both in control conditions and in neurons treated with Abeta42 oligomers (b). NMDA receptors contribute to Ca^{2+} oscillations in control (a) where APV inhibits the peak of Ca^{2+} transients. This effect is reduced in neurons treated with Abeta42 (b). The inhibition of somatic action potentials with TTX abolishes the Ca^{2+} oscillations as well (a). c) Bar graph showing that Abeta42 (light grey bar) significantly reduces the effect of APV with respect to control (dark grey bar). d) APV inhibits spontaneous firing of hippocampal network. This effect is reduced by Abeta42 (e). f) Bar graph summarizing the inhibition of spontaneous firing frequency induced by APV in control (dark grey bar) and in neurons incubated with Abeta42. The inhibitory effect is significantly depressed by Abeta42. g) Bar graph summarizing the potentiating effect of APV on network synchronization measured by considering the cross correlation probability. Although APV inhibits the average firing frequency (f) it increases network synchronization more markedly in neurons treated with Abeta42 than in control.

Fig.6

a) In control neurons cadmium together with APV completely abolishes intracellular Ca^{2+} oscillations. b) In control neurons nifedipine (light grey trace) reduces by 38% the total Ca^{2+} current (black trace). c) Bar graph showing the almost proportional halving of total, non-L type and L-type Ca^{2+} currents induced by incubation of neurons with Abeta42 (light grey bars) when compared to control conditions (dark grey bars). d) Similarly to what observed in control neurons, nifedipine (light grey trace) inhibits by 43% the total Ca^{2+} current (black trace) after incubation with Abeta42. e) Chart summarizing the main results obtained: Abeta42 increases intracellular Ca^{2+} concentration of primary cultured hippocampal and inhibits neuronal network excitability by mainly increasing the amount of Ca^{2+} released through RyRs that in turn inhibits glutamatergic AMPA dependent synapses through activation of BK channels. Finally, a further contribution to firing inhibition is given by the decreased Ca^{2+} influx through NMDARs and VGCCs induced by Abeta42.

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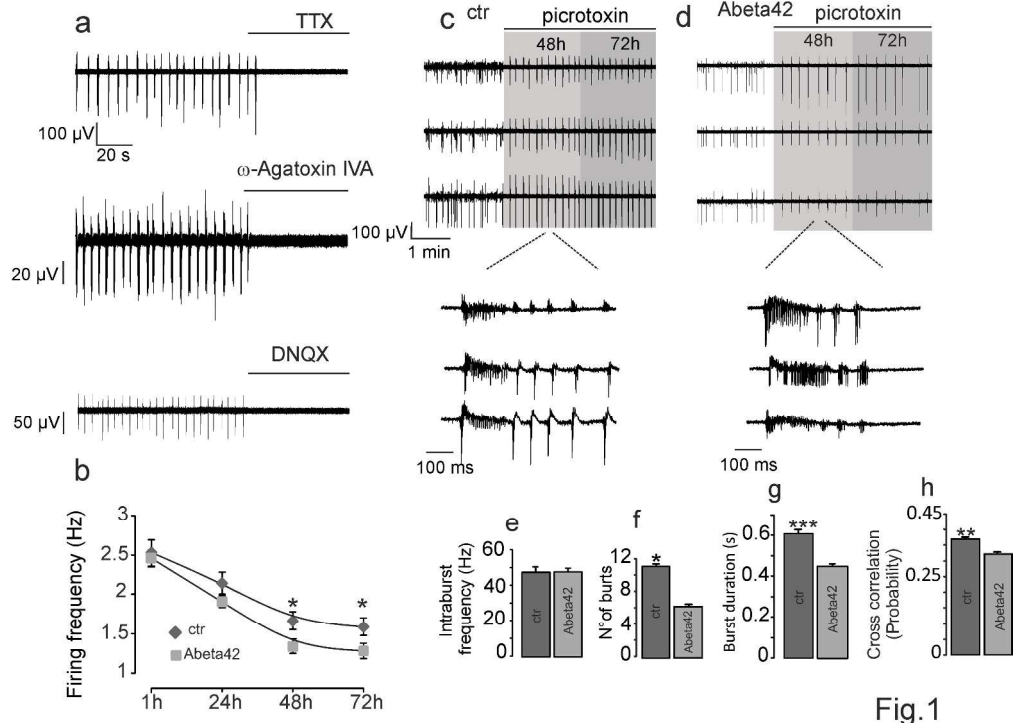


Fig.1

278x201mm (300 x 300 DPI)

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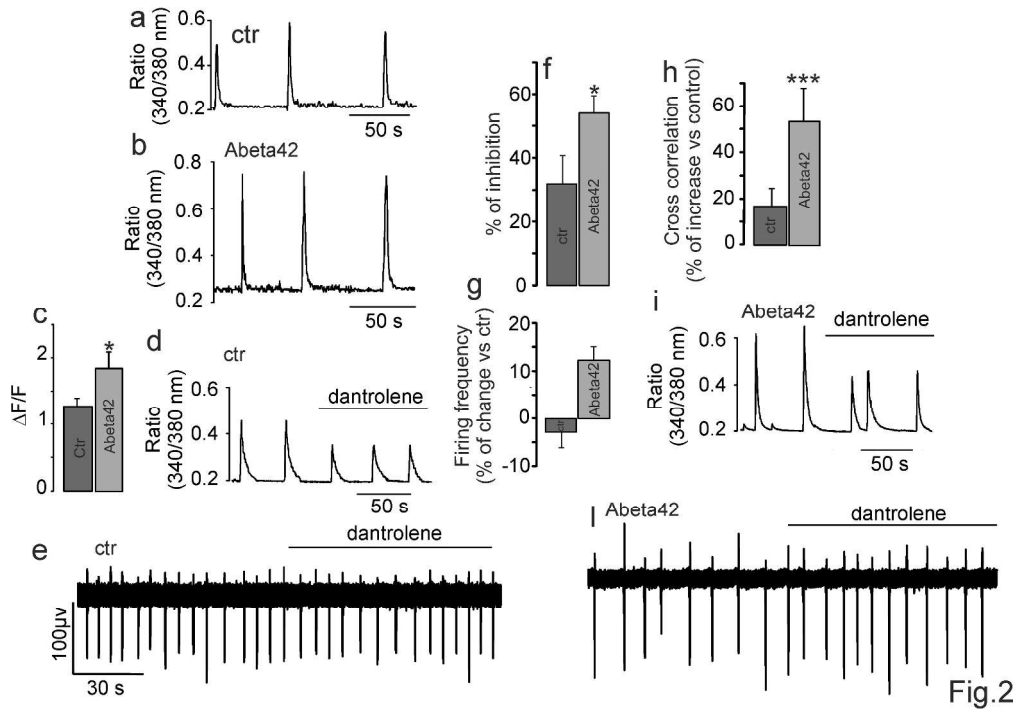


Fig.2

284x199mm (300 x 300 DPI)

Review

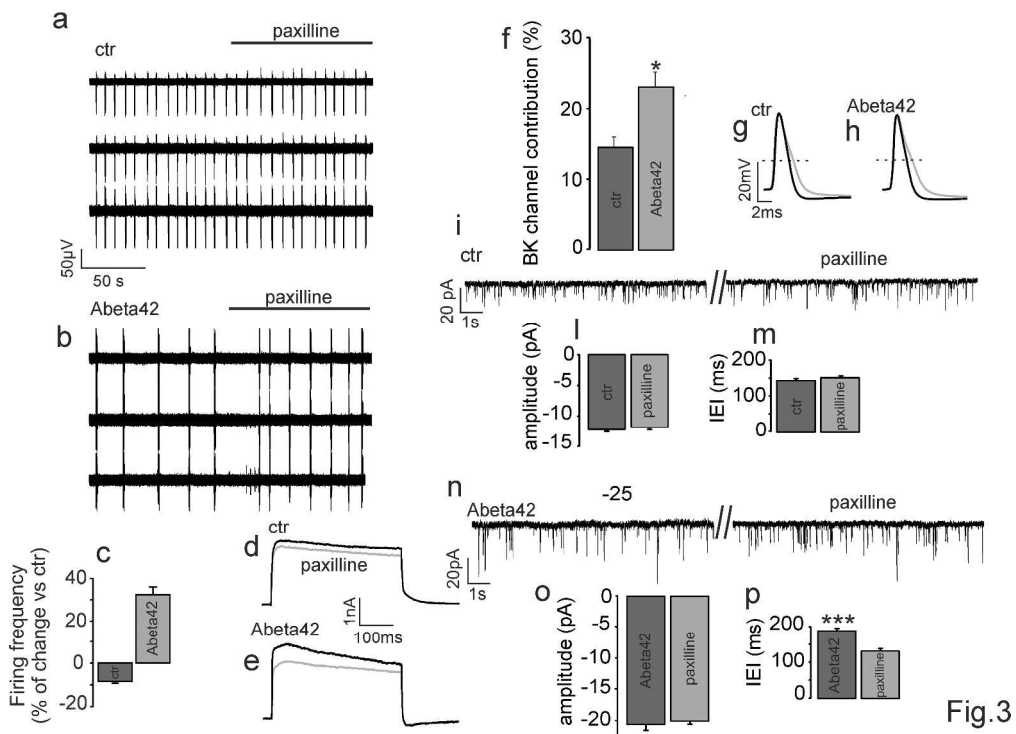


Fig.3

Fig.3

284x206mm (300 x 300 DPI)

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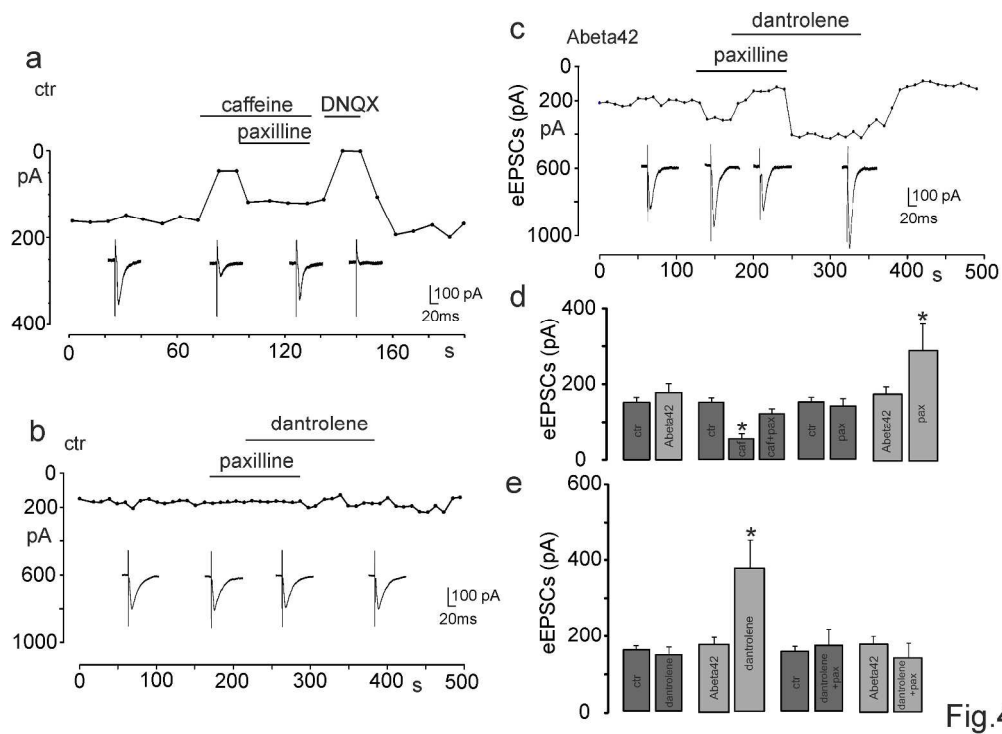


Fig.4

Fig.4

273x197mm (300 x 300 DPI)

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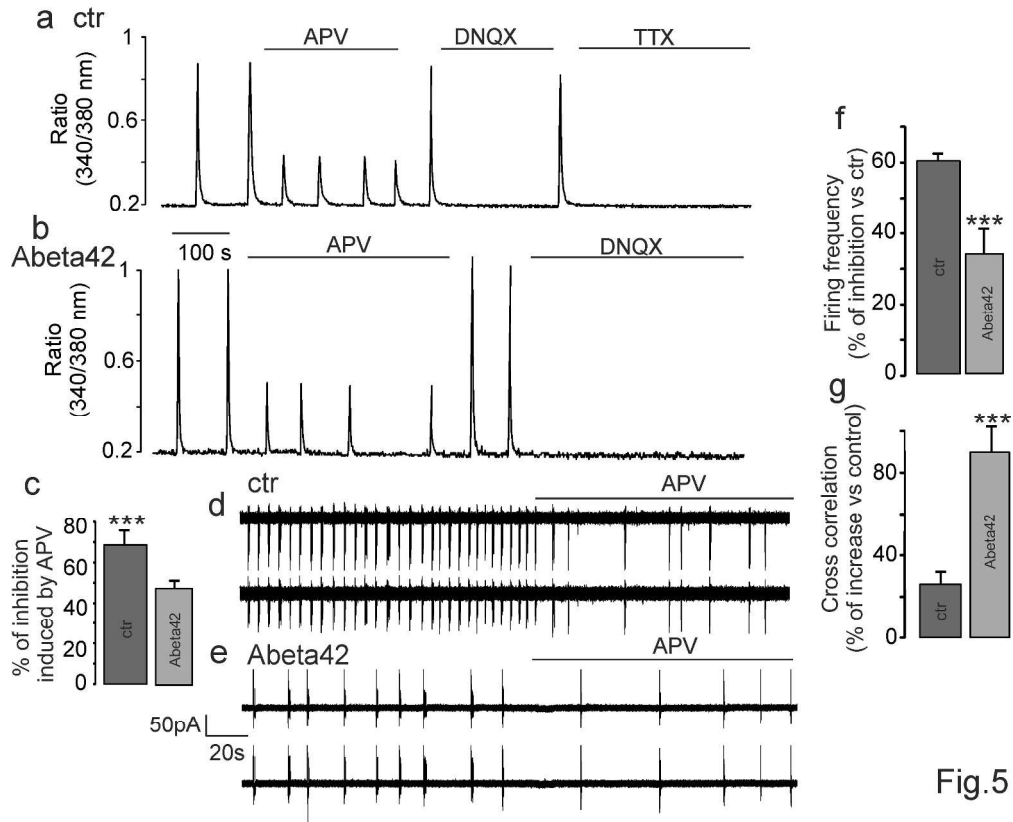


Fig.5

255x207mm (300 x 300 DPI)

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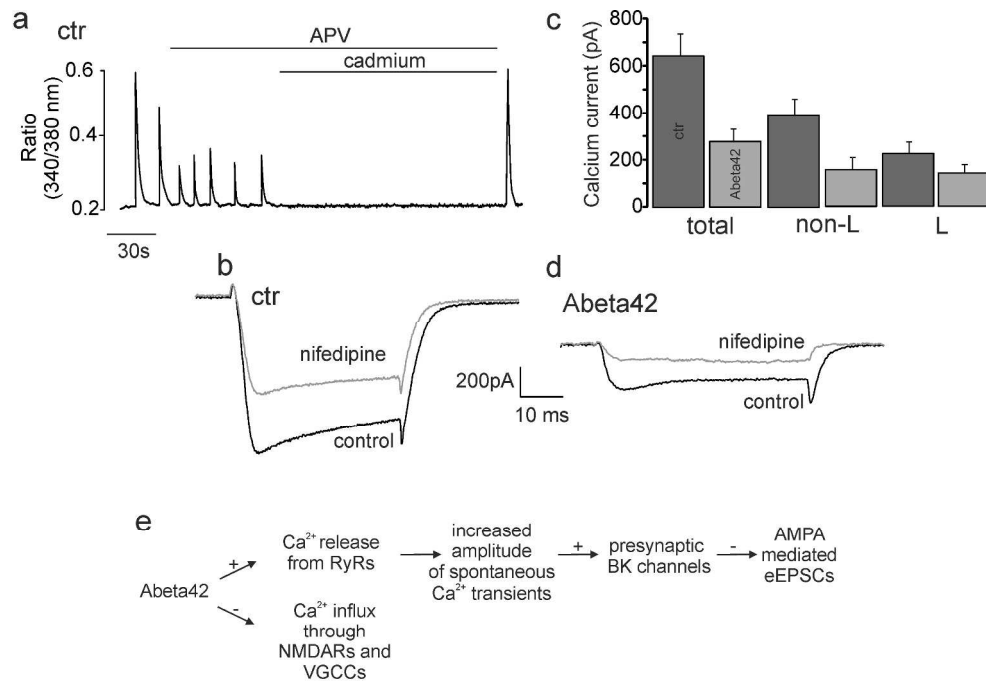


Fig.6

Fig.6

279x202mm (300 x 300 DPI)

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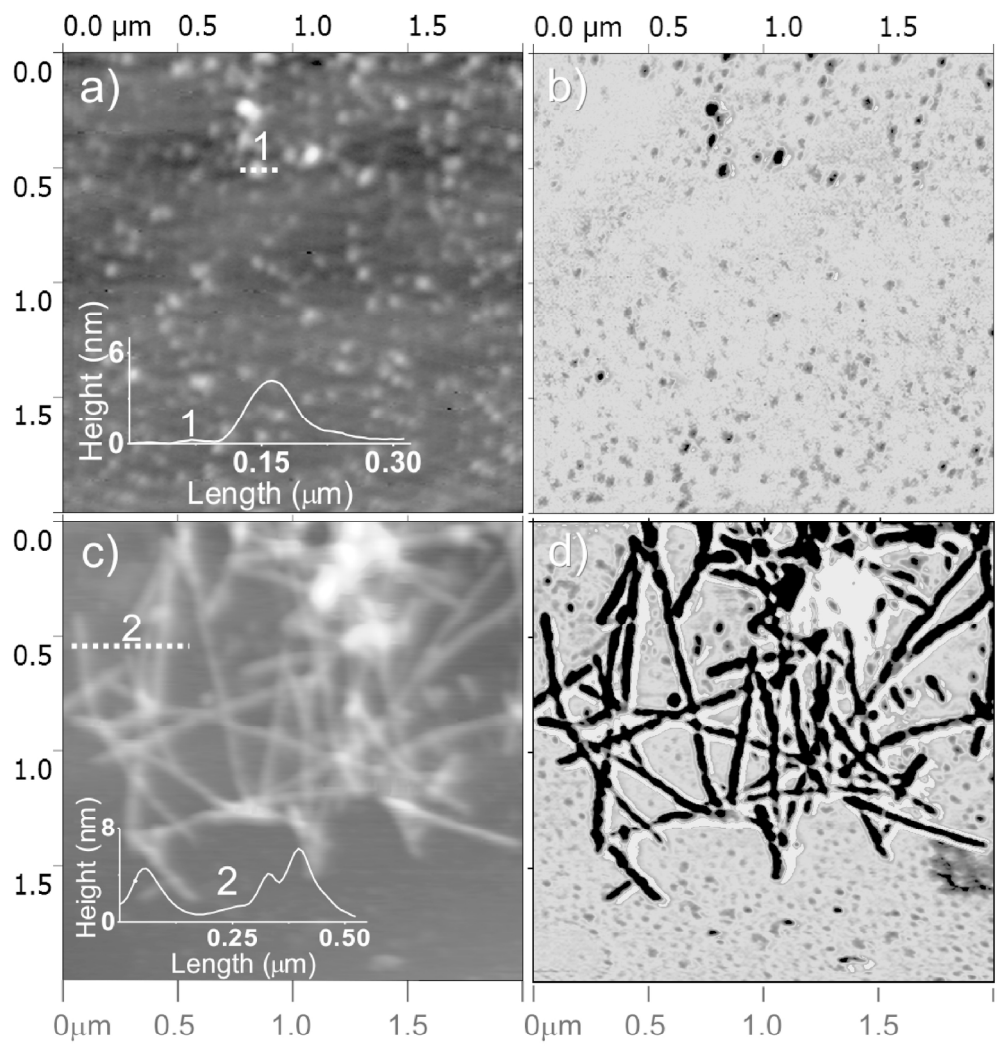


Fig. S1

Fig. S1

176x199mm (300 x 300 DPI)

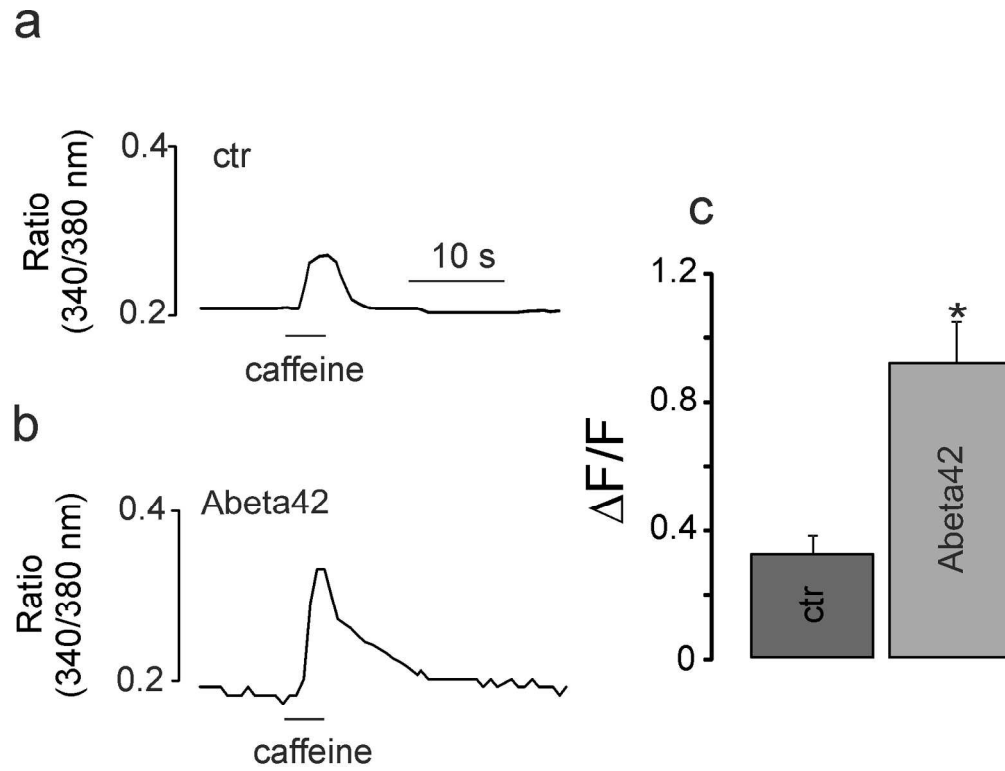


Fig. S2

Supplementary Figure S2

163x145mm (300 x 300 DPI)

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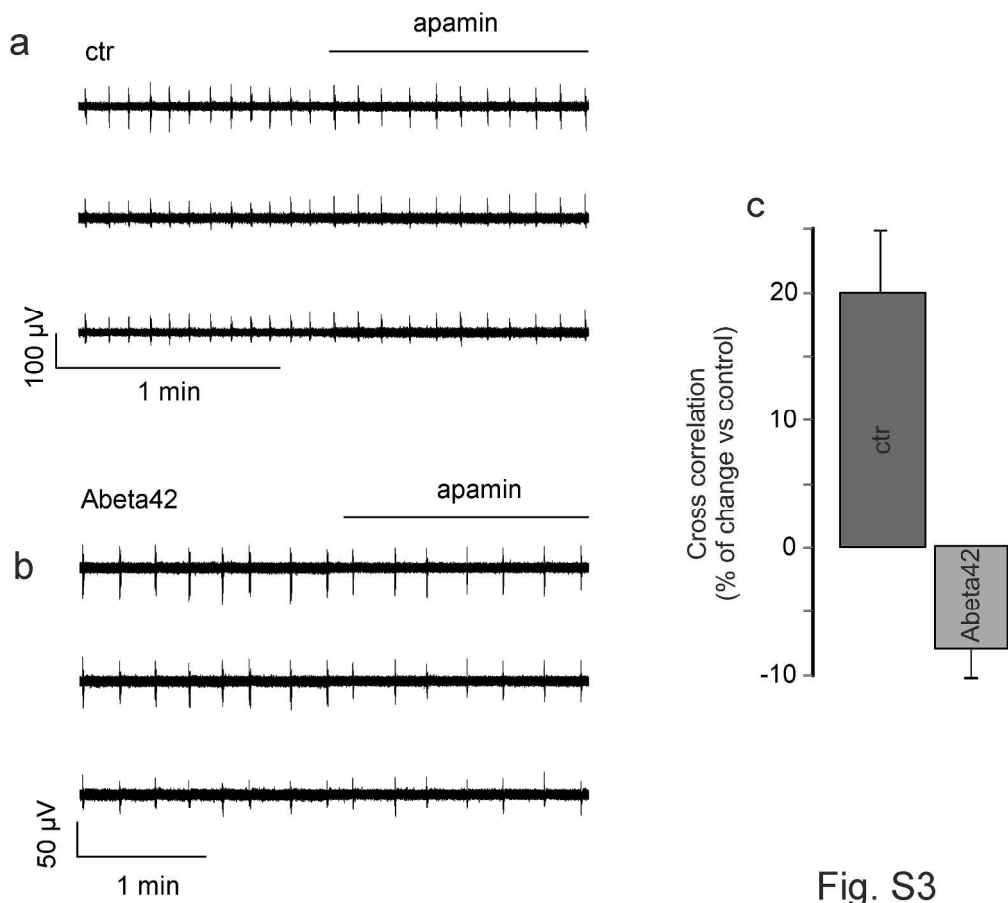


Fig. S3

Supplementary Figure S3
221x198mm (300 x 300 DPI)



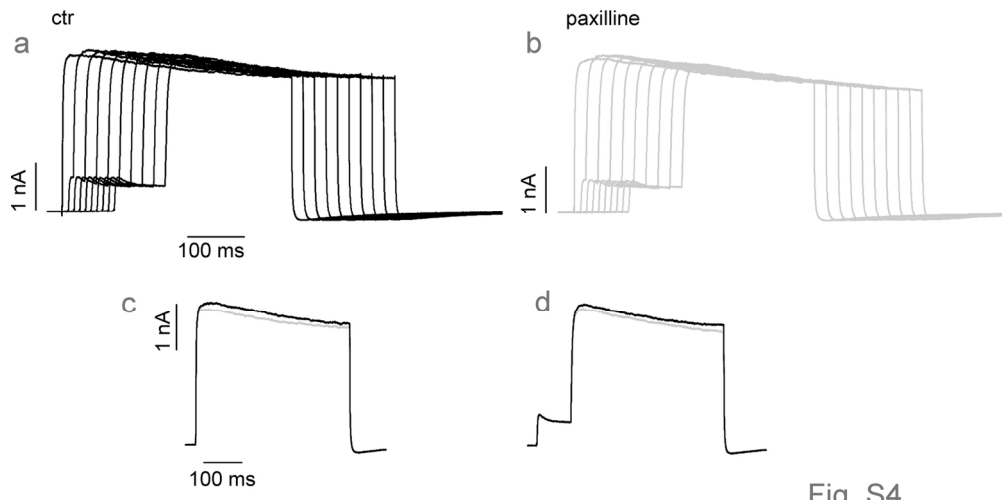


Fig. S4

Supplementary Figure S4

141x71mm (300 x 300 DPI)

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3 Supplementary Fig. S1

4 **a, b** 2x2 μ m AFM images of Abeta42 after 24h at 4°C (a: topography, b:phase signal) and **(c, d)**
5 after 48h at 37°C (c: topography, d:phase signal). Height profile selected along two dotted lines (1,
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8 2) are shown in **(a)** and **(c)**.
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3 Supplementary Fig. S2.

4
5 **a)** Effect of caffeine on intracellular Ca^{2+} concentration in control neurons. **b)** In neurons previously
6 incubated with Abeta42 caffeine stimulates Ca^{2+} release as well. This effect is potentiated when
7 compared to that observed in control neurons **c)** Bar graph summarizing the higher effect of
8 caffeine on intracellular Ca^{2+} concentration in neurons treated with Abeta42 (light grey bar) with
9 respect to control (dark grey bar).
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3 Supplementary Fig. S3.

4
5 **a)** Three representative recording traces from the same MEA showing the lack of significant effect
6 of SK channels block induced by apamin on spontaneous firing in control neurons. **b)** When
7 apamin is added to neurons previously incubated with Abeta42 it decreases the spontaneous firing
8 of hippocampal network. **c)** Bar graph summarizing the effect of apamin on network
9 synchronization by measuring the crosscorrelation in control (dark grey bar) and in neurons
10 incubated with Abeta42 (light grey bar). While apamin increases cross correlation in control
11 neurons, the opposite is observed in the presence of Abeta42.
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3 Supplementary Fig. S4.
4

5 (a) Voltage clamp recording of total outward K^+ current activated in control neurons by applying ten
6 consecutive Ca^{2+} preloading stimuli at -10mV of increasing duration (from 0 ms to 90 ms) before
7 depolarizing neurons to + 80 mV. b) BK channel contribution was estimated by administrating
8 paxilline (1 μ M). c, d) Overlapped traces from figure a (black traces) and b (gray traces) showing
9 that the amount of BK channel activated current is similar when neurons are directly depolarized to
10 + 80 mV (c) or preloaded with Ca^{2+} for 90 ms (d)
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