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Electrophysiological Functions of Intracellular Amyloid β in Specific for Cultured Human Neurones and its Impairment Properties

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Abatract

Prevailing role of intracellular amyloid β (iAβ) in Alzheimer's disease (AD) initiation and progression attracts more and more attention in recent years. To address whether iAβ induces early alterations of electrophysiological properties in cultured human primary neurons, we delivered iAβ with adenovirus and measured the electrophysiological properties of infected neurons with whole-cell recordings. Our results show that iAβ induces an increase in neuronal resting membrane potentials, a decrease in K^+ currents and a hyperpolarizing shift in voltage-dependent activation of K^+ currents. These results suggest the electrophysiological impairments induced by iAβ may be responsible for its neuronal toxicity.

Key words: Aβ, Intracellular Amyloidb, Alzheimer's Disease, Human Neurons, Electrophysiology, Impairment

1. Introduction

As one of the neurodegenerative diseases, Alzheimer's disease (AD) is characterized by intracellular neurofibrillary tangles (NFTs), extracellular senile plaques (SPs) as well as massive synaptic and neuronal loss[1-19]. The major components of SP in AD brains are amyloid β $(Aβ)_{1–40}$ or $(Aβ)_{1–42}$ [19]. Under the physiological conditions, Aβ is generated from amyloid precursor protein (APP) in the endoplasmic reticulum and the Golgi pathway, and secreted to the extracellular environment[8,14,21,28]. In addition, the involvement of endocytic pathways with endosomal-lysosomal processing is also indicated in Aβ generation[18]. However, recently, the accumulation of cytosolic intracellular Aβ $(iAβ)_{42}$ has been observed in more than one system. In the autopsy samples, $iAβ_{42}$ significantly accumulates in the pyramidal neurons of the hippocampus and the entorhinal cortex and in Purkinje cells in mild cognitively impairment (MCI) and AD patient brains[10,11,13,23,25,27,31]. The $iAβ_{42}$ accumulates in the multivesicular bodies in presynaptic and especially postsynaptic compartments[1,30]. Such accumulation may be associated with abnormal synaptic morphology before SP formation[27]. Similar accumulations of $iAβ_{42}$ also occur in neurons of Down's syndrome[5] and muscle cells in inclusion body myositis individuals[3], two degenerative disorders other than AD associated with amyloid abnormal depositions. Intracellular $Aβ_{1-42}$ accumulation appears earlier than plaque formation[11,13,25,31]. Furthermore, accumulation of $Aβ_{42}$ is reported in cell culture system[34]. In the transgenic animal models, iAβ accumulation precedes NFT formation in APP/PS1 double mutant mice[32]. Using neuronal specific promoter NF-L, $Aβ_{1–42}$ expressed intracellularly in the neurons of transgenic mice induces dramatic cell loss[20]. The results of direct delivery of Av peptides into human neurons by microinjection show that $iAβ_{1–42}$ induces a rapid cell death within 24 h after injection selectively in human neurons in primary cultures, but not in human astrocytes or the other cell lines tested[35]. Taken together, the above evidence shows that iAβ is significantly associated with neuronal cell loss and probably precedes NFT and SP formation.

To address whether iAβ induces early changes of firing pattern in human neurons; we delivered iAβ with adenovirus and measured the electrophysiological properties of infected neurons with whole cell recordings. The results show that iAβ induces an increase in neuronal resting membrane potentials, a decrease in K^+ currents and a shifted current-voltage curve of K^+ channels.

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2. Experimental Section

Primary cultures of human neurons were described before[9]. Briefly, neurons were prepared from 8-12-week-old fetal brains following the protocol approved by the Peking University Medical School Institutional Review Board (IRB00001052-0572). Fresh fetal whole brain tissues were cleaned in phosphate buffered saline (PBS) and then dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA), which was then inactivated by 10% decomplemented fetal bovine serum (FBS, HyClone, Logan, UT). The mixture was triturated through pipette to make a homogenous mixture. After filtering, the pellet was washed once by PBS and once by Dulbecco's modified Eagle's medium (DMEM) in Earle's balanced salt solution containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2mMl-glutamine, 0.1% dextrose, 1× antibiotic Pen-Strep (all from Invitrogen, Carlsbad, CA) with 5% FBS. Cells were then plated on poly-l-lysine (Sigma, St. Louis, MO) coated plates or glass cover-slips at the density of 3×106 cells/ml. In general, the cultures contain 90-95% neurons and 5-10% astrocytes [34]. Neurons were incubated at 37°C in DMEM with 5% FBS and with 5% circulating CO2. Medium was changed every 48 h. Cells were treated for experiments at around 10 days in culture. Primary rat hippocampal neuron culture was done as described before[7].

Intracellular $A\beta_{1-42}$ cDNA were sub-cloned from pEGFP-N3 into pAdTrack with BglII and XhoI digestion. Adenovirus was packaged in HEK293 cells and the infectious particle was measured as 2×106/mL (MOI = 1.33). The purified virus supernatant was added to cell culture medium for 24 h.

For adenovirus packaged Aβ detection, 5 μL virus sample was incubated with 95 μL TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and 1 μl proteinase K (20 mg/mL) overnight at 37°C. Following incubation, the sample was mixed with 100 μL of phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0) gently, then centrifuged and collected the supernatant. The supernatant was incubated with 1/10 volume of 3M sodium acetate and 2.5-fold volume of 100% ethanol overnight at -20°C. After centrifuged, discard supernatant and washed the precipitation by 1ml cold 75% ethanol. Centrifuged again and dissolved the precipitation in 20 μL TE buffer. PCR was performed with the above DNA preparations (2 μL) as templates and with specific primer sequences of Aβ gene. PCR amplification consisted of 35 cycles (94°C, 45°C, and 72°C each for 1 min) with the initial cycle for Taq activation (95°C for 10 min). PCR product and molecular weight marker were loaded into 1.5% agarose gel. After stained with ethidium bromide, the DNA bands were visualized under UV light.

For microinjection of $A\beta_{42-1}$ peptide (H-3976, Bachem, Torrance, CA), thin-walled Borosilicate glass capillaries (OD 1.0 mm, ID 0.5 mm) with microfilament (MTW100F-4, World Precision Instrument, Sarasota, FL)were pulled with a Flaming/Brown Micropipette

Puller (P-97, Sutter, Novato, CA) to obtain injection needles with a tip diameter of ~0.5 μm. Microinjections were performed in the cytosol of each cell using the Eppendorf Microinjector FemtoJet and Eppendorf Micromanipulator (Eppendorf, Hamburg, Germany). Human neurons were injected with 25 fl/shot at an injection pressure of 100 hPa, a compensation pressure of 50 hPa, and an injection time of 0.1 s. The diluted peptides were injected with 100 μg/mL Alexa488 (Molecular Probes, Eugene, OR) as a fluorescent marker to recognize the injected cells.

For Aβ detection, cells were permeablized and blocked with 10% donkey serum at room temperature, followed by incubation with anti-Aβ antibody 6E10 (Signet, Dedham, MA) in PBS-Triton at 4°C for 24 h. Cy3-conjugated donkey anti-mouse antibody was used as the secondary antibody. The nuclei were then staining by Hoechst 33258 (1 μg/mL, Sigma, St. Louis, MI) for 15 min in dark. For immunostaining of neuronal specific marker tubulin III, primary anti-tubulin III as applied and the nuclei were stained with Hoechst 33258 (1 μg/mL, Sigma, St. Louis, MI) for 15 min in dark. The cover slips were mounted with Immunon-TM mounting medium (Shandon, Pittsburgh, PA) onto glass slides. The results were analyzed by using fluorescence microscope (Olympus BH2-RFCA, Olympus, Tokyo, Japan) with digital camera (Olympus DP70 Digital Microscope Camera, Olympus). Secreted $A\beta_{1-42}$ in culture medium was measured 24 h after infection of human neurons by enzyme-linked immunosorbent assay (ELISA) kit for human $A\beta_{1-42}$ (H21656, ZHBio, Beijing, China) according to the description of the manufacturer.

For electrophysiological recordings, infected neurons

(24 h after infection) were patched in the whole cell voltage-clamped configuration. Patch pipettes were pulled on a P97 Flaming-Brown micropipettes puller (Sutter Instruments, Novato, CA) from borosilicate glass capillaries, then fire-polished to a resistance of 4-8 MΩ. The bath solution contained 130 mM NaCl, 5.4 mM KCl, 1 mM $MgC1_2$, 2 m $MCaC1_2$, 10m MHEPES and 25 mM d-glucose (pH 7.35). The electrodes were filled with 120 mM KCl, 2 mM $MgC1_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, 20 mM d-glucose and 2 mM Na2ATP (pH 7.25). After obtaining tight seals (>1 GΩ) by applying negative pressure, whole cell configuration was formed by disrupting the cell membrane. Patch clamp recording used an EPC-10 double amplifier with Pulse software (HEKA Instruments, Germany). Raw signals were low-pass filtered at 2 kHz. The neurons were held at 70 mV, then depolarized from 60 mV to 100 mV with 10 mV increments and each pulse lasted 300 ms. The leak currents and liquid junction potential were compensated by the recording software. Membrane capacitance (*C*m), input resistance (*R*input) and current amplitudes were obtained and analyzed using pulse software. The resting membrane potentials were tested in the current-patch mode.

All data are presented as means±S.E.M. Statistical significance (*p<0.05, **p<0.01 or ***p<0.001) among groups was determined by two-way analysis of variance (ANOVA) and two-tailed student's *t*-test. The Sheffe's test was applied as a *post hoc* for the significant difference shown by ANOVA.

3. Results and Discussion

To determine whether Aβ cDNA was inserted into the adenovirus vector successfully, PCR was performed to confirm the insertion of Aβ fragment in pAdTrack vector (Fig. 1A). Primary human neuronal culture (7 days in culture) contains around 90% of neurons indicated by neuronal marker tubulin III (Fig. 1B). During the transfection of primary cultured human embryo brain neurons or rat hippocampal neuorns, the infected neurons was marked by expression of EGFP, which can be identified under phase contrast and fluorescent microscope for patch clamp recordings (Fig. 1C, D and H). Successfully infected EGFP positive neurons were also positive for Aβ staining in human neurons (Fig. 1F and G). Since the antibody used to detect Aβ (6E10) also recognize human APP, rat hippocampal primary cultures were also infected with virus packaged with intracellular $Aβ_{1-42}$. Immunostaining with the same antibody still shows positive reaction confirming that $Aβ_{1-42}$ is expressed intracellularly (Fig. 1I and J). The effects of infected intracellularly located Aβ to endogenous Aβ secretion were estimated by ELISA. There is no significant difference between EGFP vector infected neurons and Aβ construct infected neurons on secreted $Aβ_{1-42}$, although Aβ infection tended to increase secreted $Aβ_{1-42}$ levels.

Basic membrane electrophysiological properties were examined in neurons infected with $iAβ_{1-42}$ constructs or microinjected with $iAβ_{42-1}$ control peptides. In $iAβ_{1-42}$ infected group, the resting membrane potentials (35.1±6.3mV) increase significantly compared with reversed peptide $Aβ_{42-1}$ (69.8±6.0mV; p<0.01) and control group (77.5±6.4mV; p<0.001) (Fig. 2B). The mean K^+ current density in $Aβ_{1-42}$ infected group (71.5±10.6 pA/pF) also decreases significantly compared with $Aβ_{42-1}$ treatment (118.2±5.1 pA/pF; p<0.05) and controls (127.2±18.0 pA/pF; p<0.01) (Fig. 2D). But the mean capacitances and the input resistances did not change significantly (Fig. 2A and C).

Whole-cell outward K^+ currents in the treated neurons were recorded in the presence of tetrodotoxin (TTX, 1 μM). With depolarizing voltage steps from -60 mV to +100 mV from a holding potential of -70 mV, large outward currents were elicited and increased (Fig. 2E). To exclude the influence of cell size for analysis, currents were converted to current density divided by membrane capacitance. After recording, current-voltage relationship was plotted with the means of peak K^+ currents. There is significant difference between the peak amplitudes of K^+ currents in $Aβ_{1-42}$ infected cells obtained from -60 mV to +100 mV compared with $Aβ_{42-1}$ injected neurons or untreated neurons (p<0.05) (Fig. 2F). The voltage-dependent activation of K^+ currents were obtained by converting the currents at different test voltages to the relative conductance (G/G_{max}) and fitting with the Boltzmann equation fraction = $\{1+\exp(V_{1/2} V)/k\}^1$. The voltage of half-maximal activation ($V_{1/2}$) and the slope factor (k) were obtained from the fitting curves. The voltage-dependent activation of K^+ currents shows a hyperpolarizing shift of $Aβ_{1-42}$ infected cells (6.67±2.59 mV) compared to control (14.35±1.99 mV) or $Aβ_{42-1}$ injected cells

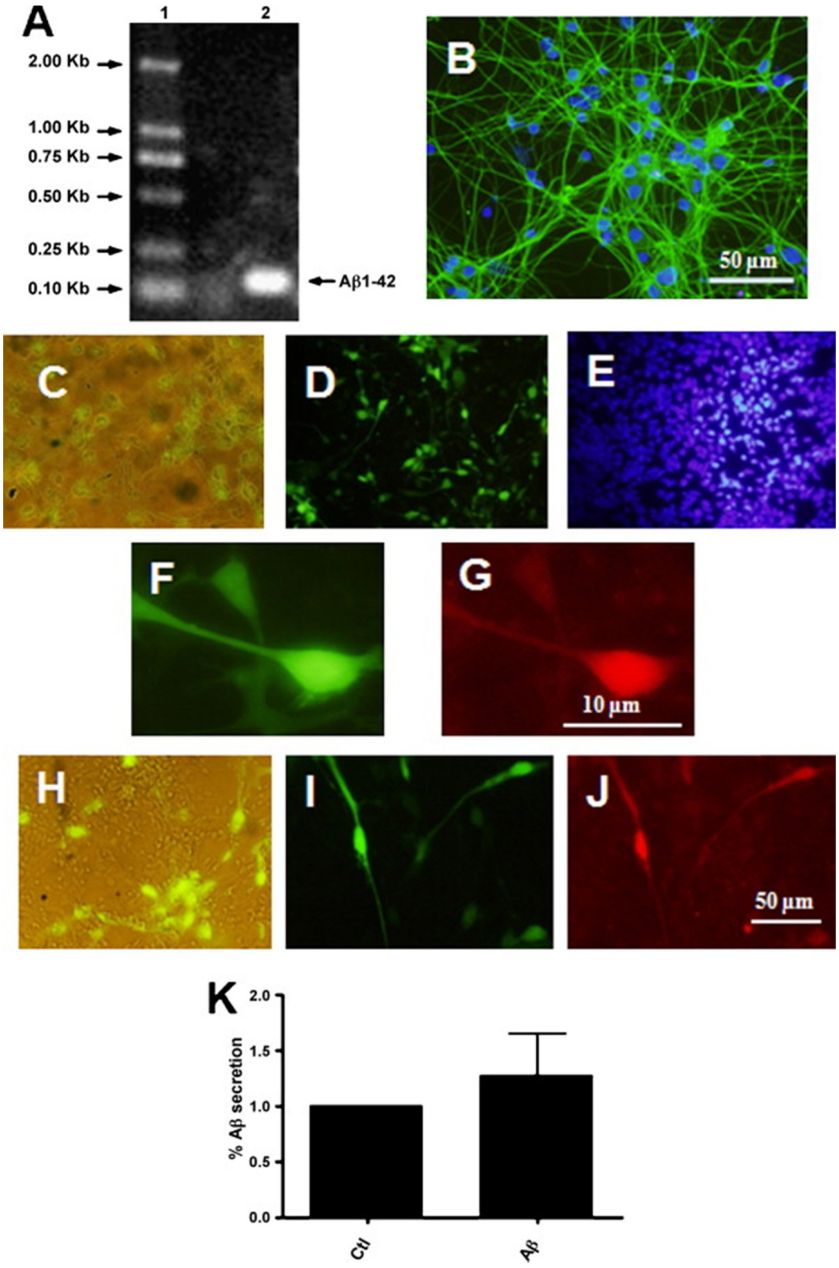

Fig. 1. Human primary neurons are infected with intracellularly expressed $A\beta_{1-42}$. (A) Electrophoresis of PCR confirms the Aβ DNA cloned into recombinant pAdTrack vector. Agarose gel (1.5%) electrophoresis of PCR amplified Aβ DNA sequence. Lane 1: DNAmarkers (D2000, Tiangen Biotech CO., LTD.); Lane 2: PCR amplification of Aβ DNA (126 bp). (B) Neuronal marker tubulin III (green) staining shows that around 90% cells in human primary culture are neurons. (C) Infected EGFP positive human neurons are recorded by patch clamp. (D) EGFP positive cells showing successful infection. (E) Hoechst staining showing total cell population. (F and G) EGFP positive human neurons are also positive for anti-Aβ antibody 6E10. (H) Infected EGFP positive rat neurons under phase contrast view. (I and J) EGFP positive rat neurons are also positive for anti-Aβ antibody 6E10. (K) After infected with intracellular Aβ construct, the secreted $A\beta_{1-42}$ was measured by ELISA. There is no significant difference between EGFP infected (Ctl) and Aβ-infected groups. Data represent means±SEM (n=3). Scale bars: B-E and H-J: 50 μm; F and G: 100 μm.

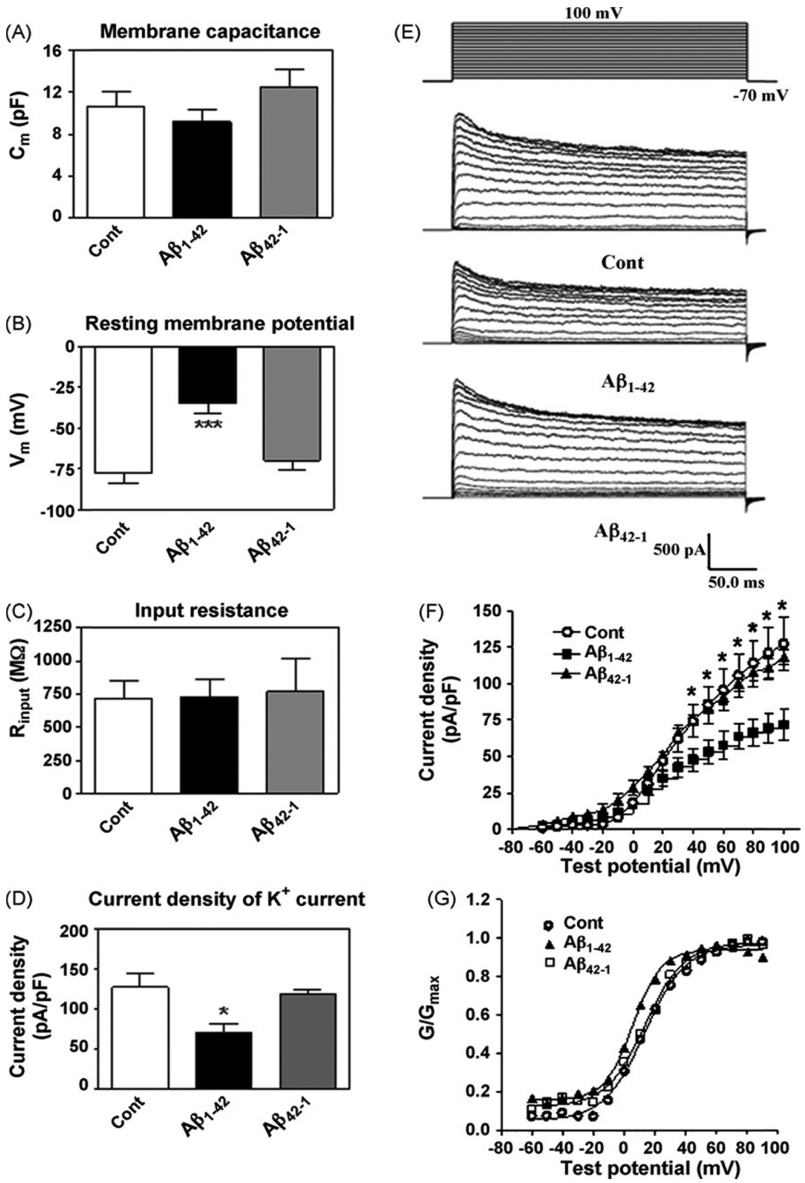

Fig. 2. Intracellular $A\beta_{1-42}$ induces changes in electrophysiological properties in human neurons. Recordings were made in human neurons treated with infected $A\beta_{1-42}$ or microinjected $A\beta_{42-1}$ compared with the control. Although the mean capacitances (A) and the input resistances (C) do not alter, with $A\beta_{1-42}$ treatment, the resting membrane potentials (B) and mean K^+ current density (D) decrease significantly compared with $A\beta_{42-1}$ and control groups ($p<0.01$). (E) Typical current curves of whole cell K^+ current obtained by depolarizing voltage potential from -60 to 100 mV in control or Aβ treated cells. (F) Current-voltage relationship for the means of peak K^+ in control (○), $A\beta_{42-1}$ (□) and $A\beta_{1-42}$ treated cells (■). Membrane currents were obtained by depolarizing voltage steps from -60 mV to +100 mV from a holding potential at -70 mV with 10 mV increasing steps. Significant differences are observed between the peak amplitudes of K^+ currents in $A\beta_{1-42}$ infected cells obtained from 60 mV to 100 mV compared with $A\beta_{42-1}$ injected neurons or untreated neurons ($p<0.05$). (G) Voltage-dependent activation of K^+ current is expressed as the relative conductance (G/G_{max}) at different voltage steps. The K^+ current activation curve has a hyperpolarizing shift in $A\beta_{1-42}$ infected cells compared to control or $A\beta_{42-1}$ injected cells. Data were acquired from seven neurons in each preparation and three independent primary culture preparations. Cont: Control. Data represent means±SEM. *$p<0.05$, ***$p<0.01$, $A\beta_{1-42}$ *vs.* Cont.

(14.03±1.24 mV; $p<0.05$). However, the slope factors have no significant changes between $A\beta_{1-42}$ group and others (Fig. 2G).

Neurons demonstrate morphological and electrophysiological changes in Aβ-mediated neurotoxicity and degeneration. Dysfunction of neuronal excitability is mainly investigated in rodent neurons and cell lines by application of synthetic Aβ extracellularly[6,12,15,33]. Little is known about the neuronal electrophysiology induced by cytosolic iA_ accumulation. In cultured human primary neurons, we find that resting membrane potential increases significantly in $iA\beta_{1-42}$ group compared with controls, whereas the other cell membrane electrophysiological properties do not change remarkably. The membrane potentials and the excitability of neurons are largely regulated by K^+ channels; for example, the outward K^+ current is mainly responsible for membrane repolarization[26]. In the present study, $iA\beta_{1-42}$ reduces the amplitude of outward K^+ currents in cultured human neurons, which is consistent with the previous reports that application of extracellular Aβ inhibits K^+ currents in cultured septal or hippocampal neurons and increases neuronal membrane excitability[6,12,15,33].

Conclusion

Our results suggest that the blockage of outward K^+ currents by $iA\beta_{1-42}$ may be one of the reasons for membrane depolarization in Aβ-containing neurons in AD brains. To explore the mechanism by which $iA\beta_{1-42}$ depresses outward K^+ currents, we examined whether the activation of voltage-gated K^+ channels was altered by $iA\beta_{1-42}$. Interestingly, $iA\beta_{1-42}$ produces a hyperpolarizing shift in the voltage-dependent activation of K^+ currents compared to controls. The inhibition induced by $iA\beta_{1-42}$ on outward K^+ currents is voltage-dependent, since the degree of inhibition increases progressively along with the depolarization. It is possible that $iA\beta_{1-42}$ interacts with the cytosolic domains of K^+ channels to induce inhibition.

A previous report shows that extracellular application of Aβ reduces outward K^+ currents and then induces a large increase of Ca^{2+} influx in the distal dendrites of rat hippocampal neurons[6]. Aβ is suggested to trigger imbalance of cellular Ca^{2+} homeostasis by activating voltage-sensitive Ca^{2+} channels[2,22,29] or forming transmembrane cation-selective channels[16,17]. Others suggest that persistent blockade of K^+ channels induced by Aβ results in cellular Ca^{2+} overloading and initiate neuronal dysfunction in rat hippocampus cells[24]. Based on the previous findings and our results, it is possible that the suppression of iAβ on outward K^+ currents contributes to iAβ cellular toxicity.

There is argument that in the present study, the expressing construct contains only $A\beta_{1-42}$, which is not generated by processing and trafficking of APP. It is reported that the signal sequences within APP, such as NPXY motif in the C-terminus, are important for Aβ generation[4]. Therefore, such construct is argued for its specific toxicity. However, in this study, the reverse peptide $A\beta_{42-1}$ does not affect electrophysiological properties of human neurons compared with $A\beta_{1-42}$ suggesting that the toxicity of $A\beta_{1-42}$ is specific. To examine if the intracellular Aβ delivered alters endogenous Aβ secretion, the amount of $A\beta_{1-42}$ in culture medium was detected by ELISA and compared between EGFP vector infected and Aβ infected groups. The data show that there is no significant difference between two groups, although Aβ infected cells show more variation on secreted Aβ.

Taken together, this is the first report of electrophysiological property impairments induced by iAβ in human primary neurons. The results of the present study contribute to our knowledge toward the understanding of iAβ neuronal toxicity and the development of iAβ-based AD treatments.

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; ANOVA, Analysis of variances; Aβ, amyloid β; eAβ, extracellular amyloid β; iAβ, intracellular Amyloid β; DMEM, Dulbecco's modified Eagle's medium; ELISA, Enzyme-linked Immunosorbent assay; FBS, fetal bovine serum; MCI, mild cognitively impairment; NFT, neurofibrillary tangle; PBS, phosphate buffered saline; SP, senile plaque; TTX, tetrodotoxin.

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