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RESEARCH ARTICLE

# The effect of apolipoprotein E4 on synchronous neural interactions in brain cultures

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**Abstract** In a previous study, we assessed the synchronous neural interactions (SNI) in a developing neural network in brain cultures on multielectrode arrays (Christopoulos et al. in J Neural Eng 9:046008, 2012). Here, we report on the effects of apolipoprotein E4 (apoE4) on these neural interactions. We carried out six experiments (five using rodent brain cultures and one using neuroblastoma cultures) in which we recorded local field potentials (LFP) from 59 sites for several days in vitro under the following conditions. In one experiment, we added to the culture media triglyceride (TG)-rich lipoproteins from a human subject with the apoE4/4 genotype, whereas in the other experiments, we added recombinant human apoE4. We found that SNI in the apoE4-treated cultures had higher coefficient of SNI variation, as compared to control cultures. These findings further document the role of SNI as a fundamental aspect of the dynamic organization of neural networks (Langheim et al. in Proc Natl Acad Sci USA 103:455–459, 2006. doi:10.1073/pnas.0509623102; Georgopoulos et al. in J Neural Eng 4:349–355, 2007) and extend the effect of apoE4 on SNI (Leuthold et al. in Exp Brain Res 226:525–536, 2013) across different brain species (human, rodents), apoE source (TG-rich lipoproteins, recombinant), neural signals (MEG, LFP), and brain network (intact brain, developing brain in vitro). To our knowledge, this is the first study of the effects of apoE4 on neural network function in vitro.

**Keywords** Apolipoprotein E4 · Brain culture · Synchronous neural interactions · Local field potentials

## Introduction

ApoE4 has been associated with various aspects of brain function and disease. The mechanisms of action of apoE4 in the brain are only partially understood and encompass various levels of reference. At the gross disease level, apoE4 is a known risk for Alzheimer's disease (Nalbantoglu et al. 1994), is involved in early onset of Alzheimer's disease neuropathology in Down's syndrome (Prasher et al. 1997), adversely affects the sequelae of traumatic brain injury (Mahley and Huang 2012a), affects susceptibility, clinical type and progression rate in multiple sclerosis (Høgh et al. 2000), and is associated with higher symptom severity in posttraumatic stress disorder (Peterson et al. 2015). At the cellular morphologic level, apoE4 has been shown to have a negative effect on neurite outgrowth in developing neural cultures (Nathan et al. 1994; MacNabb et al. 1998). Finally, at the dynamic neural network level, apoE4 affects neuronal interactions in the healthy brain by reducing their synchronicity and increasing their variability (Leuthold et al. 2013). In the present study, we sought to extend these last findings to developing brain cultures in vitro.

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## Materials and methods

### Neural cultures

*Rodent brains*

Cells were isolated by dissociation from cortical tissue of embryonic day 18 (BrainBits, Springfield, IL) of rat or mouse (strain C57B6) brains and plated on multielectrode arrays (MEAs). Details of the experimental design are given in Table 1. Cells were cultured using serum-free media (see Christopoulos et al. 2012 for details).

*Neuroblastoma cultures*

Neuro-2A cells were maintained in modified Eagle's medium (MEM) containing 10 % fetal bovine serum, and 1 % penicillin–streptomycin, and kept at 37 °C in a humidified incubator containing 5 % $CO_2$. Cells were passed every 2–4 days when they reached 80–90 % confluence using trypsin to harvest the cells. For the experiments, cells were plated at a concentration of 10,000–12,500/mL on plastic wells or poly-ornithine-coated glass slides for 6–10 h in serum-containing media. After this attachment period, wells were changed to a serum-free media (SFM), composed of neurobasal media, 1 % N-2 supplement, and 0.5 mM L-glutamine. Cultures were plated on six MEAs and treated as follows. In three control MEAs, nothing was added, beyond the media, whereas in three treatment MEAs, human recombinant apoE4 was added at a concentration of 10 μg/mL (see Table 1).

### Human subject

*Isolation of human postprandial TG-rich lipoproteins*

We used apoE associated with postprandial TG-rich lipoproteins derived from a paid human volunteer of apoE4/4 genotype 4.5–5 h following ingestion of a fatty meal "shake" containing 70 g corn oil/$M^2$ of body surface (Georgopoulos et al. 1994). The protocol for TG-protein acquisition was approved by the relevant institutional boards, and informed consent was obtained from the subject prior to fatty meal ingestion according to the Declaration of Helsinki. To avoid lipoprotein degradation and oxidation, the blood sample was collected in a tube containing EDTA (1 mg/mL) and placed on ice. Plasma was separated by ultracentrifugation at 10 °C (2000 rpm) and a cocktail containing 1 mg/mL DTPA, 0.02 mg/mL chloramphenicol, 12 % e-amino-n-caproic acid, 5 % glutathione, 1 % thimerosal, and 1 % BHT. All isolations were performed under aseptic conditions within 48–72 h of harvesting of plasma using an SW 28 rotor according to the method of Lindgren et al. (1972) as modified by Redgrave and Carlson (1979). This method required the creation of a salt density gradient and the isolation of the subfractions by ultracentrifugation. For that purpose, plasma was adjusted to $d = 1.1$ g/mL by adding KBr. NaCl–KBr solutions $d = 1.065$, followed by $d = 1.02$ and $d = 1.006$ containing EDTA, were layered successively over the plasma. The condition of ultracentrifugation for $S_f$ 20–400 was $183.2 \times 10^6$ g × min. At the end of the ultracentrifugation, the TG-rich lipoproteins were aspirated from the top of the tube (5 mL). Our recovery was >90 %. Isolated lipoproteins were characterized, dialyzed, and filtered before incubation with the cells.

*ApoE genotyping*

DNA sample from the human participant above was genotyped using PCR amplification followed by restriction enzyme digestion (Reymer et al. 1995). Each amplification reaction contained PCR buffer with 15 mmol/L $MgCl_2$ ng amounts of genomic DNA, 20 pmol apoE forward (5N TAA GCT TGG CAC GGC TGT CCA AGG A 3N) and reverse (5N ATA AAT ATA AAA TAT AAA TAA CAG AAT TCG CCC CGG CCT GGT ACA C 3N) primers, 1.25 mmol/L

**Table 1** Parameters of the various experiments (see text for details)

| Experiment | Treatment | Species | Dose (μg/mL) | MEA | DIV | Recording days | *N* | Total |
|---|---|---|---|---|---|---|---|---|
| A | apoE4/4 | Rat | 6.375 | 3C, 4T | 23 | 21 | 63C, 84T | 147 |
| B | HR apoE4 | Rat | 10 | 4C, 5T | 22 | 22 | 88C, 110T | 198 |
| C | HR apoE4 | Neuroblastoma (mouse) | 10 | 3C, 3T | 25 | 6 | 18C, 18T | 36 |
| D | HR apoE4 | Mouse | 10 | 4C, 4T | 44 | 19 | 72C, 72T | 144 |
| E | HR apoE4 | Mouse | 10 | 4C, 2T | 27 | 8 | 24C, 16T | 40 |
| F | HR apoE4 | Rat | 10 | 4C, 4T | 12 | 4 | 16C, 16T | 32 |

*apoE4/4* triglyceride-rich lipoproteins from a healthy apoE4/4 homozygote human subject, *HR apoE4* human recombinant apoE4 (see text for details), *Rat* dissociated cortical cells from E18 rat brain, *Mouse* dissociated cortical cells from E18 mouse C57B6 brain, *C* control, *T* treatment, *Collection days* were distributed along days in vitro (DIV) at regular intervals, *N* number of data points = MEA × collection days

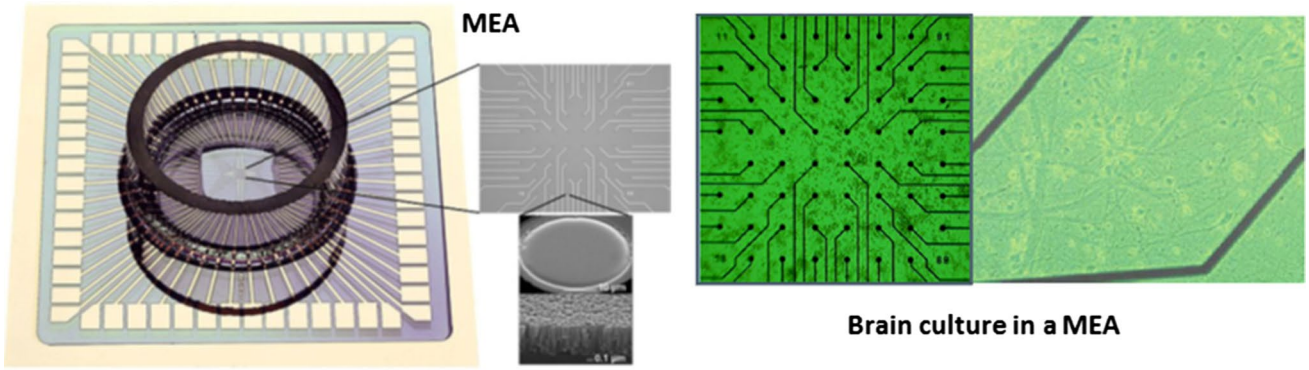


**Fig. 1** A picture of MEA used in these experiments (http://www.multichannelsystems.com/) (*left panel*) and photographs of cell cultures in it and two magnifications (*right panel*)

of each deoxynucleotide triphosphate, 10 % dimethylsulfoxide, and 0.25 μL Amplitaq DNA polymerase. Reaction conditions in a thermocycler included an initial denaturing period of 3 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C; followed by 32 cycles of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C; and a final extension of 1 min at 95 °C, 1 min at 60 °C, and 3 min at 72 °C. PCR products were digested with *HhaI* and separated on a 4 % agarose gel which was stained with ethidium bromide. Known apoE isoform standards were included in the determination.

### Multielectrode arrays (MEAs)

A MEA (Multi Channel Systems, model MEA120-2-System, Reutlingen, Germany) consists of an array of 60 electrodes embedded on a flat surface surrounded by a circular wall that creates a well around the electrodes (Fig. 1). The electrodes are titanium nitride disks, 30 μm in diameter, arranged in an 8 × 8 square array with four missing corners. They are spaced at 200-μm intervals and are attached to gold leads that connect to the 60-channel head-stage amplifier. With two sets of head- and second-stage amplifiers available, we recorded from a pair of MEAs at a time.

### Experimental design

Details of the experimental design for each of the six experiments are given in Table 1. In experiment A (Table 1), human TG-rich lipoproteins were added to the culture media at an apoE4 concentration of 6.4 μg/mL. In all the remaining experiments (B–F, Table 1), human recombinant apoE4 (Leinco Technologies, St. Louis, MO) was added to developing brain cultures at a concentration of 10 μg/mL. We plated equal number of control and treatment (apoE4) MEAs in all experiments, but several MEAs became infected and had to be discarded. The actual numbers of MEAs used in different experiments are given in Table 1.

### Neural recordings

Neural recordings began on the third days in vitro (DIV) of brain cultures (experiments A, B, and D–F in Table 1) and after 18 days of culture of neuroblastoma cells (experiment C in Table 1). In a recording session, MEAs were removed from the incubator and placed on preheated (37 °C) stands (the head-stage amplifiers) within an enclosed Faraday box to restrict light and eliminate external electrical noise. Following a 2-min stabilization period, electrical activity was recorded simultaneously from all electrodes for 1 min at a sampling frequency of 10 kHz, digitized by a 12-bit A/D converter, and stored on a hard drive for off-line processing. After each recording session, MEAs were returned to the incubator and kept there until the next recording session. Electrical signals were further amplified (×1200) in the frequency range of 1–3000 Hz by a second-stage amplifier.

### Data preprocessing and analysis

Local field potential (LFP) activity was derived from the data by applying a second-order band-pass Butterworth filter at 0.7–170 Hz to reject low and high frequencies outside the LFP range. The filtered time series were then downsampled to 1 kHz for further analysis. Thus, the data from each electrode comprised 60,000 time samples. LFP time series were prewhitened using a (25, 1, 1) ARIMA model (Box and Jenkins 1970), as described previously (Christopoulos et al. 2012), and the innovations (residuals) retained. The adequacy of the ARIMA model was assessed by evaluating the autocorrelation function (ACF) of these innovations time series. For that purpose, we applied Priestley's formula (Priestley 1981, p. 340) to calculate the approximate two-sided 5 % critical values for the autocorrelation coefficient $\widehat{\rho}(\text{lag})$ as $\pm 2\sqrt{1/N}$, where $N$ is the length of the innovations series. Then, we counted how many $\widehat{\rho}(\text{lag})$, of

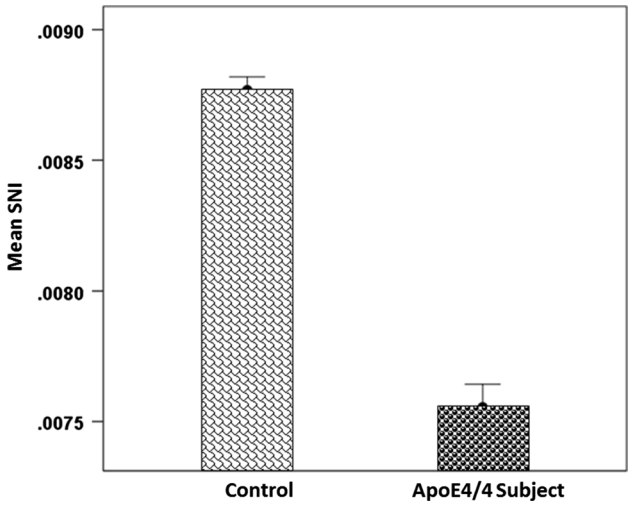


**Fig. 2** Mean SNI (±SEM) for control and TG-rich apoE4/4-treated MEA cultures, respectively. $N = 63$ and 84 MEA × DIV for control and apoE4 conditions, respectively (see Table 1 and text for details)

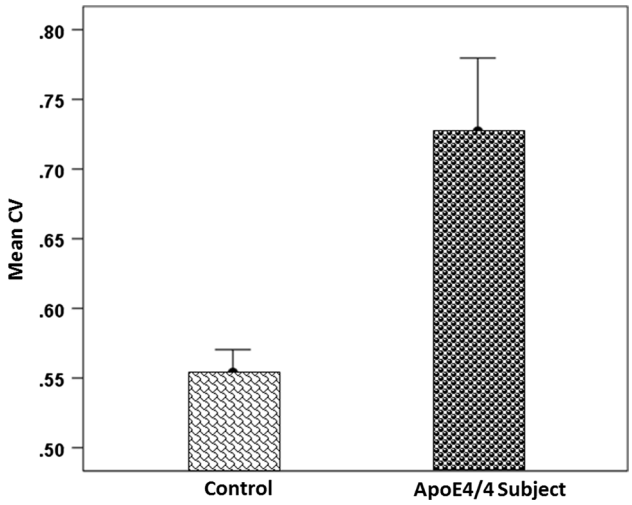


**Fig. 3** Mean CV (±SEM) for control and TG-rich apoE4/4-treated MEA cultures, respectively. $N = 63$ and 84 MEA × DIV for control and apoE4 conditions, respectively (see Table 1 and text for details)

the 25 ACF lags, exceeded that criterion. Since we would expect to find ~5 % of the lags to have $\widehat{\rho}$(lag) lying outside the critical values, even if the innovations series were white noise, we considered as adequately modeled those series for which the ARIMA (25, 1, 1) yielded innovations the ACF of which did not contain more than two lags exceeding the 5 % criteria above. Using this screening procedure, 96.5 % of the innovations series were retained for the crosscorrelation analysis. All possible pairwise crosscorrelations between the resulting innovations were computed for ±25 ms lags. Correlation coefficients, $r$, were Fisher $z$-transformed to normalize their distribution:

$$z = \mathrm{SNI} = a\tan h(r) = \frac{\ln\left(\frac{1+r}{1-r}\right)}{2}. \quad (1)$$

The coefficient of variation of $z$ was also computed:

$$\mathrm{CV} = \frac{\mathrm{SD}}{\mathrm{abs}(\mathrm{mean}\, z)} \quad (2)$$

where SD is the standard deviation of $z$. Statistical comparisons were tested using ANCOVA where group/treatment (control, apoE4) was a fixed factor and DIV was a covariate.

## Results

We found that apoE4/4 TG-rich lipoproteins (experiment A, Table 1) had a highly significant effect on lowering synchronous neural interactions (SNI) (Fig. 2; $P < 0.001$; $F$ test in ANCOVA) and increasing its coefficient of variation

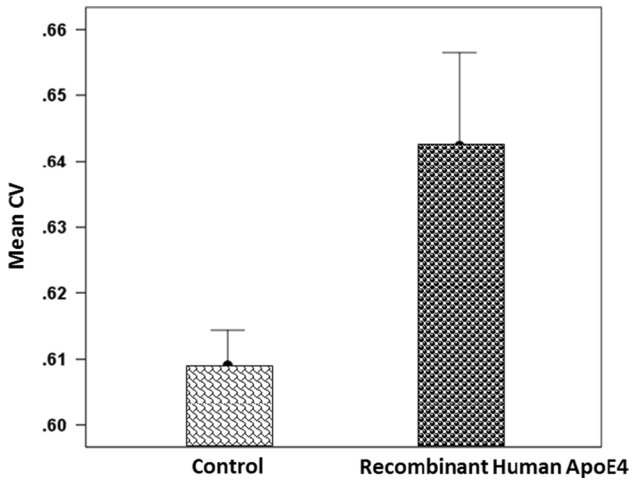


**Fig. 4** Mean SNI CV (±SEM) for control and human recombinant apoE4-treated MEA cultures, respectively. $N = 218$ and 232 MEA × DIV for control and apoE4 conditions, respectively (see Table 1 and text for details)

(Fig. 3; $P = 0.006$; $F$ test in ANCOVA). For the remaining experiments (B–F, Table 1) using recombinant human apoE4, the mean $z$ did not differ significantly between the groups ($P = 0.508$), but CV was significantly higher in the apoE4-treated cultures (Fig. 4; $P = 0.029$; $F$ test in ANCOVA), as was the SD (Fig. 5; $P = 0.004$; $F$ test in ANCOVA).

A different question concerns a comparison of SNI measures between brain cultures and the neuroblastoma culture. For that analysis, we compared mean $z$, SD, and CV between brain and neuroblastoma cultures for the experiments where human recombinant apoE was used

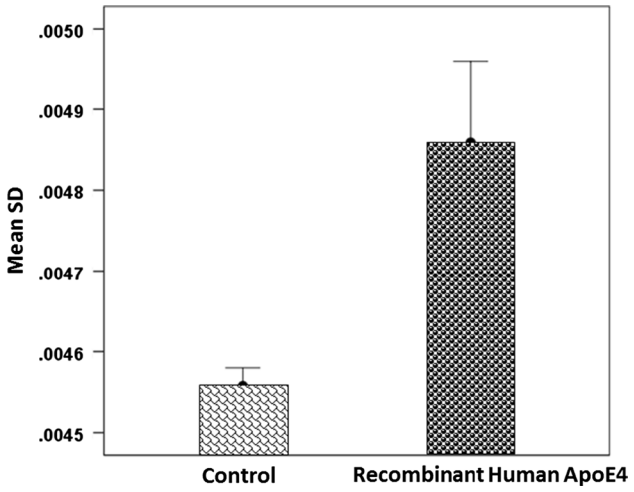


**Fig. 5** Mean SNI SD (±SEM) for control and human recombinant apoE4-treated MEA cultures, respectively. $N = 218$ and 232 MEA × DIV for control and apoE4 conditions, respectively (see Table 1 and text for details)

(B–F, Table 1). We found that mean $z$ was significantly higher ($P < 0.001$) and SD and CV significantly lower ($P = 0.003$ and $P < 0.001$, respectively) in the neuroblastoma culture (experiment C), as compared to the brain cultures (experiments B, D–F) ($F$ test in ANCOVA).

## Discussion

To our knowledge, this is the first study of the effects of apoE4 on neural network function in vitro. Our results are in accord with those reported previously regarding the increased variability of SNI associated with the apoE4 allele in cognitively healthy human subjects (Leuthold et al. 2013). In the specific case of apoE4/4 homozygotic subjects, SNI had the lowest mean and highest CV; this finding was directly replicated in the present study (Figs. 2, 3), thus extending the apoE4/4 effect from the intact human brain (Leuthold et al. 2013) to developing neural networks from rat brains and to a different neural activity measure (LFP). Although no "pure" TG-rich lipoproteins (i.e., without apoE) were used in this study, their effect on neurite outgrowth has been found to be positive (Nathan et al. 1994), and we would expect a similarly positive effect on network synchronicity, in contrast to the negative effect of apoE4 observed in both morphological (Nathan et al. 1994; MacNabb et al. 1998) and physiological settings (this study).

In keeping with the findings of Leuthold et al. (2013) in the human brain and the findings above, the increased SNI variability induced by apoE4, as assessed by the CV, was further documented here by using recombinant human apoE4 on various networks (rat brain, mouse brain, neuroblastoma cell line) (Fig. 4). It should be mentioned that the CV, being a composite measure of mean and SD, is commonly used as a standardized measure of variability across different experiments. It is noteworthy that SD was also higher in the presence of apoE4 (Fig. 5). Altogether, these findings further point to the SNI as a key measure of neural network dynamics across various experimental conditions.

The mechanism of action of apoE4 in the brain is not entirely clear. First of all, the negative effect of apoE4 on neurite outgrowth in dorsal root ganglion cell culture (Nathan et al. 1994) and neuroblastoma cell cultures (MacNabb et al. 1998) is well documented. However, it is not clear how stunned neurite growth can lead to a disrupted neural synchronicity, unless both effects reflect an overall impairment of neural network structure and function by apoE4. At the molecule structure level, apoE4 contains two arginine residues in positions 112 and 158 of the molecule (Weisgraber et al. 1981). This configuration seems to confer a lability to the apoE4 molecule which thus becomes prone to cleavage (Mahley and Huang 2012b), and when cleaved, the resulting fragments are toxic (Mahley and Huang 2012b). This toxicity of apoE4 fragments could account for the disruption of synchronicity observed in the present study. At the cellular level, toxicity is manifested in cell death, probably due to increased influx of $Ca^+$ into the cell (Veinbergs et al. 2002), since cell death is blocked in the presence of $Ca^+$ chelators or by blocking calcium channels, but not by inhibitors of intracellular calcium reserves. (Veinbergs et al. 2002). Qiu et al. (2003) also reported that human recombinant apoE3 and apoE4 the effects of recombinant human apoE4 (but not apoE3) increased the neuronal $Ca^+$ response to *N*-methyl-D-aspartate (NMDA) by 185 % as well as neurotoxicity. These effects were blocked by co-incubation with MK-801, a NMDA antagonist. Thus, excessive influx of calcium could account for the disruption of neural synchronicity, leading to the observed increased variability of neural interactions.

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**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical standard** The study protocol was approved by the appropriate institutional review board and was performed in accordance with the ethical standards outlined in the Declaration of Helsinki.

**Informed consent** The human participant provided written informed consent prior to participating in the study.

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