Directly assessing the reactivity of rat-derived microglia with scanning acoustic microscope

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1. Introduction

Two-dimensional (2D) acoustic microscopy is a useful tool to observe biological tissue using high-frequency ultrasonic waves directly. One of the advantages of this microscopy method is observing living cells without undergoing the histochemical staining process. Thus, saving time on staining and avoiding the loss of biological function of the observed sample. Scanning acoustic microscope (SAM) (AMS-50SI) developed by Honda Electronics, Toyohashi has two modes; acoustic impedance mode and sound speed mode.

In this study, we study the effects of Acetamiprid (ACE) on microglia (MG). MG plays an important role in neuronal development because it has two phases; phagocytic M1 phase and neuroprotective M2 phase. MG in the M1 phase shows ameboid form; in contrast, MG in M2 phase shows ramified form; however, by observing the form, it is merely a small significant method to identify M1 or M2. We considered off-balance of M1/M2 would induce developmental neurotoxicity.

ACE, a type of insecticide from the group neonicotinoid, mimics the nicotine chemical structure to bind to the nAChR. It has been seen as safer insecticide than organophosphate compounds; however, recently, some reports suggest otherwise. We have observed developmental alteration of cerebellar neurons, particularly the Purkinje Cell (PC) exposed to ACE on gestation day 15. The ACE-exposed pups showed PC misalignment and excessive folding between lobule V and VI in the cerebellar vermis 14 days after birth (P14), similar to ASD model animals. As MG and astroglia control neuronal alignment and survival, ACE exposure would change both the balance of M1/M2 and astrocyte development. Controversy, in well-known herbicide Glyphosate-exposed pups, PC was decreased with development.

This study investigates the identification method of M1/M2 of cultured MG derived from ACE- or Glyphosate-exposed pups via acoustic microscopy.

2. Methods

2.1 Microglial cell preparation

We cultivated glial cells using a glia-selective culture medium after exposure to ACE (40 mg/kg body weight) or Glyphosate (250 mg/kg body weight) on G15 pregnant rats cerebellum of newborn pups in Angled Neck Cell culture flask. MG was isolated from astrocytes mechanically at several days *in vitro* (DIV). For acoustic or optical observation, we used the PS-film dish (Honda Electronics Co., Ltd.) All cells were cultured in the CO_2 incubator, 37℃. Cultured cells were observed using phase-contrast microscopy daily.

2.2 Acoustic cell observation

We observed cultured MG using ultrasound microscopy with a center frequency of 320 MHz. The basic calibration is shown in **Fig 1**, whereby the signal reflected from the target is compared to the signal reflected from the reference material.

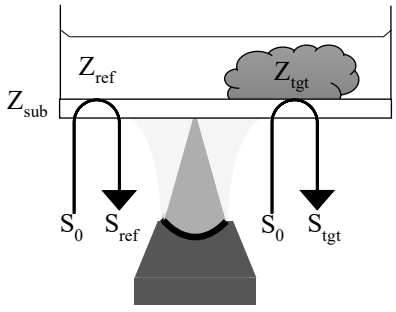

Fig. 1 Illustration of acoustic impedance observation

The signal from the target material is shown as

$$Stgt = \frac{Ztgt - Zsub}{Ztgt + Zsub} S0$$

Here, S_{tgt} and S_{ref} are signal reflections from the target and reference respectively. S_0 is the

transmitted signal and is always constant during observation. Z_{tgt}, Z_{ref} and Z_{sub} are the acoustic impedances of the target, reference, and substrate, respectively. Polystyrene film (Z_{sub} = 2.46 Nsm^{-3}) was used as substrate, while for culture medium (Z_{ref} = 1.52 Nsm^{-3}) was used as reference material.

On the one hand, the reflected signal from the reference material is shown as follows.

$$Sref = \frac{Zref - Zsub}{Zref + Zsub} S0$$

In the current system, S_0 cannot be measured. Therefore, the acoustic impedance value of the target can be calculated by formulating simultaneous equations related to the acoustic impedance Z_{tgt} and S_0 of the target substance and solving them as follows.

$$Ztgt = \frac{1 - \frac{Ztgt}{Z0}}{1 + \frac{Ztgt}{Z0}} Zsub = \frac{1 - \frac{Stgt}{Sref} \cdot \frac{Zsub - Zref}{Zsub + Zref}}{1 + \frac{Stgt}{Sref} \cdot \frac{Zsub - Zref}{Zsub + Zref}} Zsub$$

3. Results and discussion

Using SAM, isolated MG derived from the cerebellum showed a round form. In **Fig 2**, MG had the plasmalemma area around the thick cell body. MG derived from control pups had thick and thin areas in the cell body; each MG would make a bridge-like form. On the other hand, MG derived from Glyphosate-exposed pups showed the uniform intensity of the cell body forming the ameboid form.

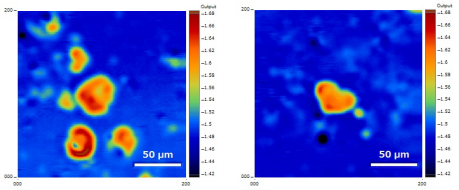

Fig. 2 Acoustic impedance images of MG with PBS as control (left) and Glyphosate (right) exposed cerebellum

Previous reports showed that when ATP receptor is expressed with ATP treatment, MG changes to M2 phase. However, when MG is at M1 phase, ATP receptor was not expressed. MG derived from ACE-exposed pups also showed a round form, and after ATP treatment, some MGs transformed ramified M2-like form. We suggest ATP signaling would induce MG to the M2 phase, but some MGs derived from pesticide-exposed pups could not accept ATP signaling.

While PC is misaligned in developing cerebellum of ACE-exposed pups, MG number of ACE-exposed pups looked increasing but not significant. Each MG formed ramified phase and would protect PC excessively. **Fig 3** shows the results of the developing cerebellum stained immunohistochemically with anti-Iba1 antibodies for MG and anti-Calbindin D28-k antibodies for PC using confocal microscopy.

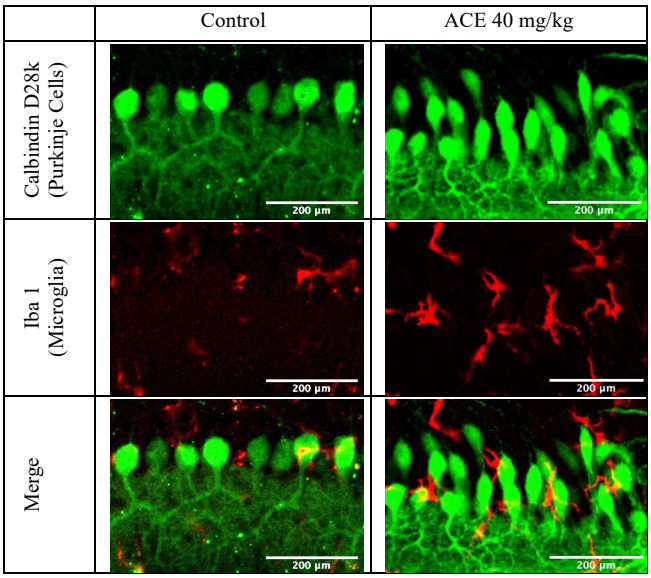

Fig. 3 PC and MG distribution in control or ACE-exposed cerebellum

Identifying the M1 or M2 phase of MG is important; however, immunohistochemical tools are insufficient for this identification because M1/M2 transition would be dynamic in living MGs. SAM observation of living MG gives us important information about M1/M2 transition and interaction between MG and neurons.

Acknowledgment

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