Evaluation of M1-microglial activation by neurotoxic metals using optimized organotypic cerebral slice cultures

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ABSTRACT — M1-microglia (neurotoxic microglia) regulate neuronal development and cell death and are involved in many pathologies in the brain. Although organotypic brain slice cultures are widely used to study the crosstalk between neurons and microglia, little is known about the properties of microglia in the mouse cerebral cortex slices. Here, we aimed to optimize the mouse cerebral slice cultures that reflect microglial functions and evaluate the effects of neurotoxic metals on M1-microglial activation. Most microglia in the cerebral slices prepared from postnatal day (P) 7 mice were similar to mature microglia in adult mice brains, but those in the slices prepared from P2 mice were immature, which is a conventional preparation condition. The degree of expression of M1-microglial markers (CD16 and CD32) and inflammatory cytokines (tumor necrosis factor-α and interleukin-1β) by lipopolysaccharide, a representative microglia activator, in the cerebral slices of P7 mice were higher than that in the slices of P2 mice. These results indicate that M1-microglial activation can be evaluated more accurately in the cerebral slices of P7 mice than in those of P2 mice. Therefore, we next examined the effects of various neurotoxic metals on M1-microglial activation using the cerebral slices of P7 mice and found that methylmercury stimulated the activation to M1-microglia, but arsenite, lead, and tributyltin did not induce such activation. Altogether, the optimized mouse cerebral slice cultures used in this study can be a helpful tool to study the influence of various chemicals on the central nervous system in the presence of functionally mature microglia.

Key words: Organotypic slice cultures, Mouse cerebral cortex, Microglia, Neurotoxic metal

INTRODUCTION

Microglia are known as immunocompetent cells and are involved in the induction of inflammatory cytokine expression in the brain in response to various stimuli (Kettenmann et al., 2013). Neuron-microglia crosstalk is crucial for neuronal dysfunction in a variety of pathological conditions (Bessis et al., 2007; Hanisch and Kettenmann, 2007). In addition, recent studies suggest that microglia-derived cytokines not only directly promote neuronal cell death, but also indirectly induce neuronal damage via astrocyte-mediated gliotransmission. For instance, the release of tumor necrosis factor-α (TNF-α) from microglia potentiated astrocytic glutamate release, which can lead to neurotoxicity (Bezzi et al., 2001). To evaluate such crosstalk among multiple types of cells in the brain, an in vitro co-culture system of neurons, astrocytes, and microglia is necessary. Organotypic brain slice cultures retain the relevant cytoarchitecture and types of cells that constitute the brain (Humpel, 2015). Therefore, molecular biology, imaging, electrophysiology, and immunohistochemical techniques are routinely used to evaluate organotypic brain slice cultures to investigate the molecular and cellular processes of neurodevelopment, synaptic plasticity, and cell death in both physiological and pathophysiological contexts (Lein et
In particular, hippocampal slice cultures have been frequently used in many studies. In contrast, cerebral cortex slice cultures are rarely used despite the many pathologies related to neuronal dysfunction in the cerebral cortex such as Parkinson’s disease, corticobasal degeneration, epilepsy, and neurotoxic chemical-induced cerebral damage (Gibb et al., 1989; Guerrini and Carrozzo, 2001; Scatton et al., 1982; Weinberger and Lipska, 1995).

Microglia derived from erythromyeloid progenitors enter the central nervous system during embryonic (E) stages E8.5-E9.5 (Xavier et al., 2014). At neonatal stages (postnatal [P] days, P0-P3), immature microglia populate the brain. Subsequently, the microglia undergo proliferation and disperse throughout the cerebral cortex at 1-2 weeks after birth, acquiring their mature ramified morphology, which involves long branching processes (Xavier et al., 2014). Mature microglia exist as resting state (M0-microglia) in the brain, and sense microscopic changes in the brain environment by their processes and transform to neurotoxic microglia (M1-microglia) (Franco and Fernández-Suárez, 2015). When M0-microglia are transformed to M1-microglia by inflammatory stimuli, the morphology of the microglia changes to amoeboid, a round shape, and the cells produce neurotoxic inflammatory cytokines e.g., TNF-α and interleukin (IL)-1β (Franco and Fernández-Suárez, 2015).

In most previous studies, the cerebral cortices of early neonatal mice were conventionally used for the organotypic brain slice cultures (Chattopadhyaya et al., 2013; Nishimura et al., 2010; Pohland et al., 2015). However, there is a possibility that the crosstalk between neurons and M1-microglia was not evaluated correctly since the microglia are still immature in this culture system. Therefore, in order to optimize the preparation methods of organotypic cerebral slice cultures, we first evaluated the activation of M0-microglia to M1-microglia by lipopolysaccharide (LPS), a representative M1-microglia stimulator (Lehnardt et al., 2002), in the cerebral slices prepared from early and late neonatal mice. Previous studies have reported that several neurotoxic metals induce the expression of neurotoxic inflammatory cytokines in the cerebrum (Escudero-Lourdes, 2016; Mitra et al., 2013; Takahashi et al., 2018; Tsunoda and Sharma, 1999). However, the involvement of M1-microglial activation in this process and in the induction of neuronal damage has not been elucidated. Therefore, we also evaluated the effect of various neurotoxic metals on the activation to M1-microglia using organotypic cerebral slice cultures.

### MATERIALS AND METHODS

#### Animals

The present study was performed in accordance with the recommendations of Regulations for Animal Experiments and Related Activities at Tohoku University. All mice used in the study were C57BL/6 mice purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were housed in plastic cages at 22 ± 2°C and a relative humidity of 55 ± 20% with a 12 hr light-dark cycle. Food (F-2, Oriental Yeast, Tokyo, Japan) and filtered tap water were provided ad libitum. Organotypic cerebral cortex slices were prepared from P2, P7, and P14 mouse pups.

#### Organotypic cerebral slice culture preparation

We optimized the previously reported preparation method of organotypic cerebral slices (Gogolla et al., 2006). Briefly, 1 hr before dissecting the mice, 1.2 mL of slice culture medium (50% Opti-MEM, 25% Hanks’ Balanced Salt Solution [HBSS], 25% heat-inactivated horse serum, 2 mM L-glutamine, 6.5 mg/mL glucose, 100 units/mL penicillin, and 100 mg/mL streptomycin) were added to each well of a six-well plate with a culture plate insert, which was prepared according to the method of Koyama et al. (Koyama et al., 2007), and incubated in 5% CO2 at 37°C. The cerebral cortices of mouse pups were dissected and quickly placed in ice-cold HBSS containing 6 mg/mL of glucose and 15 mM HEPES for 5 min. The cerebral cortices were cut into 350 µm-thick sagittal slices using a Mcllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Cambridge, UK), and incubated on ice for 30 min in HBSS containing 6 mg/mL of glucose and 15 mM HEPES. Two intact slices were placed on the culture plate insert of the six-well plate prepared as above, and maintained in 5% CO2 at 37°C. The incubation media were replaced after 24 hr and changed for every 3 days. It has been suggested that the process of preparing live brain tissue slices activates M0-microglia to M1-microglia and releases inflammatory cytokines, likely as a consequence of mechanical injury or death of some neurons during the tissue slicing procedure (Hailey et al., 1996; Dailey and Waite, 1999). Indeed, the induction of inflammatory cytokine (TNF-α and IL-1β) expression is significantly increased by the mechanical stress associated with tissue slicing; however, the induction of such expression was decreased to steady state within the pre-cultures for 4 days in the current study (data not shown). Generally, eight-well cultivations are possible from one mouse pup using the above preparation method.
Exposure of neurotoxic metals to cerebral slice cultures

After the pre-cultures of the cerebral slice cultures, culture medium was changed to fresh medium with various concentrations of chemicals. After incubation for 8 hr, the medium was discarded and the cerebral slices were washed with PBS for 2 times, and applied to the following assays.

Immunohistochemistry
The mice were dissected and the blood was removed by injection of saline to the left ventricle to the inferior vena cava. Whole brains were obtained and immersed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 days at 4°C, and further incubated in 20% sucrose in PBS for 2 days at 4°C. The fixed brains were mounted on a cryostat (Leica Biosystems, Wetzlar, Germany) using optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) and cut into 30 µm-thick sections. The cryosections were carefully mounted on glass slides and permeabilized and blocked by the buffer (0.3% Triton X-100, 10% fetal bovine serum [FBS] in PBS) for 1 hr at 22 ± 2°C. Cultured slice tissues were immersed in ice cold PBS with the hydrophilic PTFE membrane (Millipore, Burlington, MA, USA) for 1 min, and the slices were then fixed with 4% PFA in PBS for 20 min at 22 ± 2°C. After washing with PBS for 10 min, the slices were incubated in permeabilized buffer (0.3% Triton X-100, 10% FBS in PBS) for 1 hr at 22 ± 2°C.

The brain sections were then immersed in anti-Iba1 antibodies (Wako Pure Chemical, Osaka, Japan), anti-NeuN antibodies (Abcam, Cambridge, UK) or anti-GFAP antibodies (Cell Signaling Technology, Danvers, MA, USA) diluted in PBS and incubated at 22 ± 2°C for 3 hr. After two washes with PBS for 5 min, the samples were incubated with fluorescent-conjugated antibody (Alexa Fluor Plus 555, Thermo Fisher Scientific, Waltham, MA, USA) diluted in PBS for 1 hr at 22 ± 2°C. Mounting was performed with VECTASHIELD mounting medium hard set with DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Confocal microscopy (FV1000, Olympus, Tokyo, Japan) was used for image acquisition.

Measurement of mRNA levels using real-time quantitative polymerase chain reaction
The slices were removed from the culture membrane using a spatula and collected in a 1.5 mL tube. The total RNA was isolated from the tissues or brain slices using Isogen II according to the manufacturer’s instructions. Reverse transcription was performed using PrimeScript® RT reagent kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s protocol. Real-time quantitative polymerase chain reaction (qPCR) was performed using KAPA SYBR (KAPA Biosystems by Thermal Cycler Dice® [Takara Bio Inc.]) with the following primers:

- GAPDH, F:5’-AACCTTTGGCATTTGGAAGG-3’, R:5’-ACACATTGGGTTGAGGAACA-3’.
- CD16, F:5’-CCAAGACGATCTCAGGCA-3’, R:5’-TTCTGCGTTGCTGAATCCTT-3’.
- CD32, F:5’-AATCCTGCCGTTCTACTGATC-3’, R:5’-GTGTCACCAGTGCTCCTTGTGAG-3’.
- TNF-α, F:5’-CACACTCAAAAAACCAAGTG-3’, R:5’-TTTGAGATCCATGCCGTTGG-3’.
- IL-1β, F:5’-GCCCCATCTCTGATGACATCAT-3’, R:5’-AGGCCACAGGTATTTGTCG-3’.

GAPDH was used as an internal control and the data are presented as the GAPDH-corrected value.

Statistical analysis
Statistical significance was analyzed by one-way ANOVA and Tukey’s post hoc test.

RESULTS

Effects of the age of the mice used for the cerebral slice cultures on microglia maturation
We prepared cerebral slices from P2, P7, and P14 mice, since microglia mature approximately 2 weeks after birth (Xavier et al., 2014). However, the cerebral slices prepared from P14 mice did not adhere well to the culture insert and failed to culture for more than 3 days.

The microglial morphology was assessed in the cerebral slices prepared from 6-week-old mice by immunostaining using the antibody against Iba1, a microglial protein marker. As mentioned above, mature M0-microglia are ramified in basal conditions. Most of the microglia in the cerebral cortices of the 6-week-old mice were ramified M0-microglia, as previously reported (Fig. 1A). The microglial morphology in the cerebral slices prepared from P7 mice was also ramified as in the brains of the 6-week-old mice; however, almost all of the microglia in the cerebral slices prepared from P2 mice were round in shape (amoeboid) (Fig. 1B). The number of microglia in the cerebral slices prepared from P7 mice was slightly greater than that in the slices prepared from P2 mice, but the difference was not significant (data not shown). The process number of microglia and basal expression levels of M1-microglia-specific markers (CD16 and CD32) in the cerebral slice-
es of P7 mice were similar to those observed in the brains of 6-week-old mice (Fig. 1C), but were significantly lower in the cerebral slices of P2 mice. These results indicate that the amoeboid microglia observed in the cerebral slices prepared from P2 mice were not M0-microglia activated to M1-microglia, but were immature microglia, while the ramified microglia in the cerebral slices of P7 mice were similar to those observed in the brains of 6-week-old mice. In addition, it is considered that most of the microglia matured to show a morphology of the ramified type during pre-incubation of cerebral slices prepared from P7 mice for 4 days.

Fig. 1. Morphology of microglia in the cerebral cortices of adult mice and the cerebral slice cultures. (A) Cerebral cortices of C57BL6 mice (6-weeks-old) and cerebral slices prepared from P2 or P7 mice were immunostained with Iba1 antibodies. Enlarged images are provided in the lower panel. The scale bars indicate 40 µm and 10 µm in the upper and lower panels, respectively. (B) The number of microglial processes was quantified (n = 10). The data are presented as the mean ± standard deviation, *P < 0.05, **P < 0.01 vs the cerebral cortex. (C) The mRNA levels of CD16 and CD32 in the cerebral cortices of C57BL6 mice (6-weeks-old) and cerebral slices prepared from P2 or P7 mice were determined by real-time quantitative polymerase chain reaction (qPCR) (n = 3). The data are presented as the GAPDH-corrected value expressed as the mean ± standard deviation. Y-axis indicates the ratio with the cerebral cortex as 1. *P < 0.05, **P < 0.01 vs the cerebral cortex.

Effects of LPS on microglial morphology, and expression of inflammatory cytokines and M1-microglial markers in cerebral slices prepared from P2 and P7 mice

We next confirmed the extent of M1-microglial activation of microglia in P2 or P7 slices by application of the inflammatory ligand, LPS, because it specifically binds to toll-like receptor 4, which is predominantly found on surface of microglia (Lehnardt et al., 2002). The microglial morphology in the cerebral cortex slices prepared from P7 mice was transformed to amoeboid with a reduced number of processes after LPS stimulation. However, LPS did not affect the number of microglial processes in
the cerebral slices from P2 mice (Figs. 2A and B). These results indicate that morphological evaluation of activated M1-microglia is possible in cerebral slices prepared from P7 mice but impossible in slices from P2 mice.

When the cerebral slices prepared from P2 and P7 mice were stimulated with LPS, the expression levels of TNF-α and IL-1β were increased in both slices (Fig. 3A). The expression levels of CD16 and CD32 were also increased by LPS (Fig. 3B). However, the increase in the expression levels of these cytokines (TNF-α and IL-1β) and M1-markers (CD16 and CD32) in the cerebral slices from P2 mice was significantly lower than that in the slices from P7 mice. These results suggest that microglia in the cerebral slices prepared from P7 mice are more sensitive to LPS-induced activation than are those in the cerebral slices prepared from P2 mice.

**Evaluation of the effects of various neurotoxic metals on M1-microglial activation**

From the above results, cerebral slices prepared from P7 mice could be a useful tool to evaluate M1-microglia-mediated neuronal dysfunction. We first examined the cytotoxic concentrations of arsenite, lead, methylmercury, and tributyltin in a mouse microglial cell line BV2, and set the maximum treatment concentrations to non-cytotoxic concentrations; arsenite (500 µM), lead (500 µM), methylmercury (10 µM), and tributyltin (500 µM). Application of 1 or 5 µM of methylmercury to cerebral slices prepared from P7 mice resulted in induction of CD16 and CD32 expression, but the other metals did not elicit this effect at any concentration (Fig. 4). We confirmed that neuronal cell damage caused by methylmercury was not observed under this condition (data not shown). This suggests that neuronal cell damage may not be involved in the M1-microglial activation by methylmercury. However, this effect was reduced by 10 µM of methylmercury, but under this condition, slight cytotoxicity was observed (data not shown). Moreover, the microglial morphology was transformed to amoeboid by treatment with 5 µM of methylmercury or 100 µM of tributyltin (Fig. 5). These results suggest that methylmercury stimulates activation from M0-microglia to M1-microglia. However, tributyltin could not induce the expression of CD16 and CD32 even though the microglial morphology was transformed to amoeboid.

**DISCUSSION**

The results of this study suggest that microglia in mouse cerebral cortex slices prepared from P7 mice are more mature than those in the cerebral slices prepared from P2 mice.
from P2 mice. The cerebral slice cultures prepared from P7 mice are useful as an experimental model system related to the central nervous system in the presence of functionally mature microglia. In Fig. 3B, LPS induced the expression of CD16 and CD32 in the immature microglia in the cerebral slices prepared from P2 mice. This result indicates that immature microglia can also induce the expression of M1-microglia-specific markers. However,
the basal expression levels of CD16 and CD32 were significantly higher in the cerebral slices prepared from P7 mice, and were increased by LPS in the immature microglia of P2 mice but were still lower than the basal levels in the microglia of P7 mice. Although the role of CD32 in the activation of immune cells is not well characterized, overexpression or knockdown of CD16 is known to be correlated with the inflammatory responses in monocytes (Shalova et al., 2012). Therefore, the immature microglia with low expression of CD16 may be associated with the inert response to LPS. It is known that immature microglia are distinct from mature microglia with regard to certain characteristics. First, the release of inflammatory cytokines by LPS stimulation was significantly lower in immature microglia than in mature microglia (Kuwabara et al., 2003). Second, immature microglia do not express sex hormone receptors (Turano et al., 2017). Thus, cerebral slices prepared from P2 mice may be insufficient to evaluate neuronal toxicity induced by inflammatory cytokines produced by microglia, and also inappropriate for investigation of neurotoxic endocrine-induced disruption of M1-microglial activation. Our results strongly support that the present optimized method using the P7 mice will overcome the above problems. Cerebral slice cultures are expected to show similar responsiveness to in vivo, however it is not possible to easily investigate the response in each brain constituent cells. Recently, the improved culture methods for primary microglia and astrocytes that respond in vivo similarly has been reported (Bohlen et al., 2017; Foo et al., 2011). Therefore, by using such a primary cell cultures and the cerebral slice cultures in combination, it is expected that the response by LPS and various chemicals can be accurately examined in the mouse brain.

Using the cerebral slice cultures prepared from P7 mice, we found that methylmercury stimulates activation to M1-microglia. We previously reported that methylmercury induces expression of inflammatory cytokines (TNF-α, IL-1β and IL19) and CC chemokine ligands, which produced M1-microglia or mediators of microglia activation, in the cerebral cortices of mice (Iwai-Shimada et al., 2016; Kim et al., 2013; Takahashi et al., 2015, 2018). Our preliminary study also shown that minocycline, an inhibitor of microglia activation, and clodronate-liposome, a microglia depleting agent, suppressed methylmercury-induced neuronal cell death in the cerebral slices prepared from P7 mice (unpublished data). These results suggest that M1-microglial activation by methylmercury causes neuronal cell death. Therefore, it is expected that the mechanisms involved in the neuronal toxicity caused by methylmercury will be clar-

![Fig. 5. Effects of neurotoxic metals on microglial morphological change in the cerebral slice cultures. (A) Cerebral slices prepared from P7 mice were exposed to the indicated concentration of methylmercury (MeHgCl), arsenite (NaAsO₂), lead (PbCl₂), and tributyltin (TBT-Cl) for 8 hr, and immunostained with Iba1 antibodies. Enlarged images are provided in the right panel. The scale bars indicate 40 µm or 10 µm in the upper and lower panels, respectively. (B) The number of microglial processes was quantified (n = 10). The data are presented as the mean ± standard deviation. n.s.: not significant. N.D.: not detected.](image-url)
ified by elucidating the mechanisms underlying M1-microglia activation by methylmercury. However, tributyltin could not induce the expression of CD16 and CD32 even though the microglial morphology was transformed to amoeboid. Many previous studies have used tributyltin concentrations of 1-10 μM; thus, the concentration used in this experiment may have been too high (Mizuhashi et al., 2000). Moreover, it has been reported that tributyltin did not stimulate activation to M1-microglia in various cell culture models (Röhl et al., 2009). Thus, the amoeboid microglial morphology observed after application 100 μM of tributyltin may have been a different response from M1-microglial activation. To date, while there has been no report that arsenic or lead causes M1-microglial activation, it has been suggested that lead induces neuronal cell death via astrocyte activation (Struzynska et al., 2001). Our present data suggest that both metals failed to induce M1-microglial activation. Taken together, neuronal toxicity caused by both metals may be independent from M1-microglial activation. These results indicate that the optimized cerebral slice cultures can be a helpful tool to study the toxicological significance of M1-microglia activation by neurotoxic chemicals.

When M0-microglia are stimulated by various inflammatory ligands, the transcription factor Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) is activated and induces M1-microglia-related genes (Taetzsch et al., 2015). A recent study has suggested that administration of methylmercury to rats causes the induction of inflammatory cytokine expression in microglia at the dorsal root via activation of NF-κB through the Rho/Rho-associated protein kinase pathway (Fujimura et al., 2019). We also confirmed that methylmercury activated NF-κB in primary microglia and the cerebral slice cultures (data not shown). These results support that methylmercury may stimulate activation of M0-microglia to M1-microglia via NF-κB activation. Therefore, elucidation of the mechanisms involved in NF-κB activation by methylmercury in microglia may aid the elucidation of the mechanisms underlying methylmercury-induced neurotoxicity.

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Conflict of interest——The authors declare that there is no conflict of interest.

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