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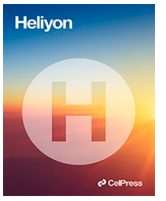

Research article

Long-lasting increases in $GABA_B$ receptor subunit levels in hippocampal dentate gyrus of mice with a single systemic injection of trimethyltin

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ABSTRACT

We have recently shown delayed increases in $GABA_B$ receptor ($GABA_BR$) subunit protein levels in the hippocampal dentate gyrus (DG), but not in the pyramidal CA1 and CA3 regions, at 15–30 days after the systemic single administration of trimethyltin (TMT) in mice. An attempt was thus made to determine whether the delayed increases return to the control levels found in naive mice afterward. In the DG on hippocampal slices obtained at 90 days after the administration, however, marked increases were still seen in protein levels of both $GABA_BR1$ and $GABA_BR2$ subunits without significant changes in calbindin and glial fibrillary acidic protein (GFAP) levels on immunoblotting analysis. Fluoro-Jade B staining clearly revealed the absence of degenerated neurons from the DG at 90 days after the administration. Although co-localization was invariably detected between $GABA_BR2$ subunit and GFAP in the DG at 30 days on immunohistochemical analysis, $GABA_BR2$-positive cells did not merge well with GFAP-positive cells in the DG at 90 days. These results suggest that both $GABA_BR1$ and $GABA_BR2$ subunits would be tardily and sustainably up-regulated by cells other than neurons and astrocytes in the murine DG at 90 days after a systemic single injection of TMT.

1. Introduction

Research on nerve tissue regeneration and transplant therapy for neurodegenerative diseases in the central nervous system is still at dawn. Alzheimer's disease (AD) is a disease in which the loss of neurons in the hippocampus, which is important for memory formation, is noticeable, resulting in a decline in cognitive function. Neural stem/progenitor cells reserve in the hippocampal dentate

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Abbreviations

AD	Alzheimer's disease
ANOVA	one-way analysis of variance
DG	dentate gyrus
FJB	Fluoro-Jade B
$GABA_BR$	$GABA_B$ receptor
GFAP	glial fibrillary acidic protein
TMT	trimethyltin
RT	room temperature

gyrus (DG), and if their, i.e. patient's, own cells can be artificially differentiated, AD should become a treatable disease. Trimethyltin (TMT), an organotin compound once used as a disinfectant and chemical stabilizer, is a neurotoxicant of which poisonings observed when humans consume it include memory loss, increased aggression, convulsions, and ataxia [1–3]. In murine brains, TMT damages the cerebral cortex, hippocampus, and olfactory bulb with particularly selective injures to granule cells in the DG [1,4]. In consistency with the loss and subsequent reconstruction of synaptic network in the DG, animals exhibited loss and regain of cognitive functions [5]. Since it proceeds in parallel to the proliferation of neural stem/progenitor cells in the dentate subgranular zone, TMT could be a useful compound to create a model for studying neural tissue regeneration at least in the DG. However, the intercellular networks that consist neural tissue are complex, so the efforts are required to collect detailed information and elucidate it.

In our previous study [6], a single intraperitoneal injection of TMT resulted in marked increases in $GABA_B$ receptor ($GABA_BR$) subunit protein levels in the DG, but not in the CA1 and CA3 regions, at 15–30 days after the administration in mice. Marked decreases were seen in protein levels of glutamatergic receptor subunits and the mature neuron marker calbindin in the DG between 2 and 30 days after the injection, in contrast, while expression profiles of $GABA_BR2$ subunit, but not $GABA_BR1$ subunit, were similar to those of the astroglial marker glial fibrillar acidic protein (GFAP) rather than the neuronal marker Tubulin-III within 30 days after the administration [6]. The view that $GABA_BR$ belongs to the G-protein coupled receptor family with seven transmembrane domains and needs to form a heterodimer between two subunits such as $GABA_BR1$ and $GABA_BR2$ for active membrane signaling is prevailing [7]. The $GABA_BR1$ subunit has been believed to have an agonist binding site with the $GABA_BR2$ subunit for a G protein binding site [7]. Since our previous data argue in favor of an idea that $GABA_BR$ subunits could be inducibly expressed in cells other than neurons after the neuronal damage by TMT, an attempt was made to evaluate expression profiles of both $GABA_BR1$ and $GABA_BR2$ subunit proteins in the DG afterward up to 90 days after the systemic administration of TMT in this study.

2. Materials and methods

2.1. Materials

TMT was purchased from Fujifilm Wako Chemical Co. (Miyazaki, Japan). Fluoro-Jade B (FJB) was obtained from Nacalai Tesque (Kyoto, Japan). Western Lightning Chemiluminescence Reagent Plus was product of PerkinElmer (Waltham, MA). IgGs were purchased from companies below: calbindin and GFAP (Cell Signaling Technology Inc., Danvers, MA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and N-terminus of $GABA_B$ receptor R2 ($GABA_BR2$) subunit (Santa Cruz Biotechnology, Santa Cruz, CA). C-terminal regions of $GABA_BR1$ and $GABA_BR2$ subunits were produced as previously described [8]. Secondary antibodies used were anti-rabbit IgG conjugated with Alexa Fluor® 555 (Cell Signaling Technology Inc., Danvers, MA), anti-mouse IgG conjugated with FITC antibody (Sigma Chemicals, St. Louis, MO) and with Alexa Fluor® 488 (Thermo Fisher Scientific Inc., Waltham, MA) for immunohistochemistry, or horseradish peroxidase conjugated anti-rabbit IgG (Dakocytomation Carpinteria, CA), anti-chicken IgY (Jackson ImmunoResearch Inc., West Grove, PA) and anti-mouse IgG (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) for immunoblot analysis. All other chemicals were of the highest purity available.

2.2. Animals

The protocol used here met the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University (K13–1, K14-1). Adult male Std-ddY mice weighing 25–30 g were housed in metallic breeding cages with a light–dark cycle of 12 h–12 h and a humidity of 55 % at 23 °C and given free access to food and water. After habituation under these breeding conditions for a week, mice were intraperitoneally administrated with TMT at 2.8 g/kg. The same volume of PBS as the TMT solution was administrated to the other mice, hereinafter collectively referred to as "Naive". By conducting a follow-up study for 90 days, three times as long as the previous report (30 days) [6], we investigated whether the effects of TMT administration had completely recovered.

2.3. Immunoblot analysis

Hippocampus dissociated from mouse brain was immediately sliced by a tissue slicer (model 51425, Stoelting, IL) with a thickness

at 500 μm for immunoblot analysis. For immunoblot analysis, the DG was dissected under a microscope and those preparations from both hemispheres were collected together and lysed by sonication in 10 mM phosphate buffered saline (pH 7.0) containing 2 % SDS together with (mM) orthovanadate (1.0), sodium pyrophosphate (10), sodium fluoride (50), EGTA (5), EDTA (5) and NaCl (100) for SDS-PAGE according to the procedures described previously [6]. Briefly, 10 μg protein aliquots were subjected to electrophoresis and migrated proteins were transferred onto PVDF membranes (Merck Millipore, Darmstadt, Germany). The membranes were incubated with 5 % skim milk for 1 h, with the desired primary antibodies for 2 h, and with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. The antibody-specific immunoreactive bands were detected on X-ray film using Western Lightning Chemiluminescence Reagent Plus. In order to confirm the data shown in our previous study [6], entirely another set of experiments was designed to validate experimental protocols in this study.

2.4. Histological analysis

For histological analysis, mice were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.), followed by perfusion via the heart with saline and then with 4 % (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were quickly removed and fixed overnight at 4 °C in the same fixative solution as above. The fixed brains were embedded in paraffin, and sagittal sections with a thickness of 2 μm were prepared using a microtome [6]. In FJB staining for degenerative neurons, 0.0002 % FJB in 0.1 % acetic acid was reacted with the section for 1 h at room temperature (RT). For immunohistochemistry, the sections were incubated with 5 % (vol/vol) goat serum for 1 h at RT, with the desired primary antibodies overnight at 4 °C, and with fluorescent dye-conjugated secondary antibodies for 1 h at RT [6].

2.5. Data analysis

Densitometric analysis for quantification of immunoreactive bands was carried out using Atto Densitograph software (Atto Co., Tokyo, Japan). Data were expressed as means ± S.E., one-way analysis of variance (ANOVA) was evaluated (for supplementary table), and statistical significance was determined using Dunnett's test.

3. Results

To confirm the neurotoxicity of TMT, immunoblot analysis was performed on lysates prepared from the DG in hippocampal slices obtained at different days up to 90 days after the systemic administration (Fig. 1). Protein levels of calbindin, a marker of mature neurons, and GFAP, a marker of astrocytes, significantly decreased and increased respectively within 10 days after TMT as shown previously [6]. These significant alterations individually remained even at 30 days after the administration, but returned to each control level by 90 days later. By contrast, both $GABA_BR1$ and $GABA_BR2$ subunit protein levels significantly and sustainably increased in the DG from 10 to 90 days after TMT. Accordingly, expression profiles of both $GABA_BR1$ and $GABA_BR2$ subunits did not well

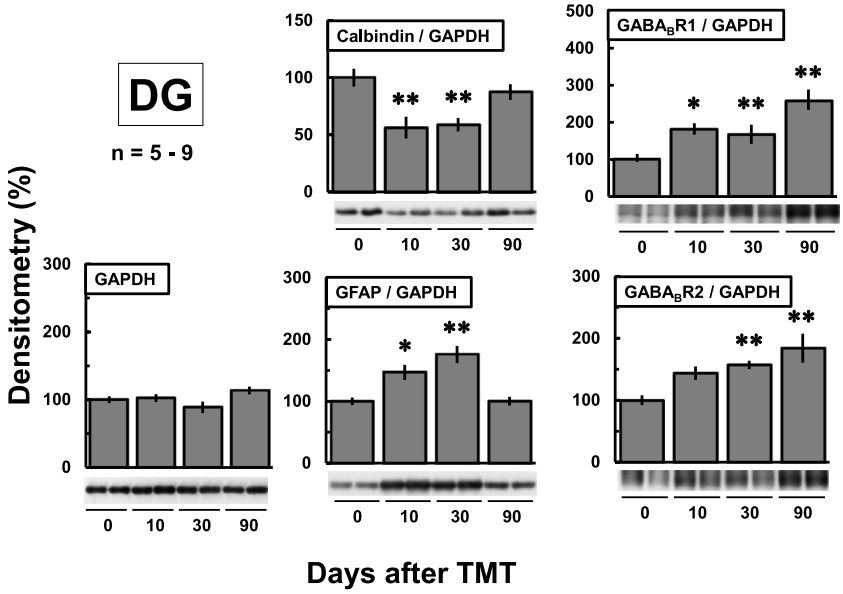

Fig. 1. Levels of protein expression in DG after TMT. Animals were given TMT (2.8 mg/kg, i.p.) and decapitated at different days after the administration. Hippocampus was subjected to dissection of the DG, which was lysed for determination of calbindin, GFAP, GAPDH, $GABA_BR1$ and $GABA_BR2$ on immunoblotting analysis. Experiments were independently repeated 5–9 times. *$P < 0.05$, **$P < 0.01$, significantly different from the untreated control "0" (Dunnett's test).

coincide with those of either calbindin-positive neurons or GFAP-positive astrocytes in the hippocampal DG at 90 days after the single administration of TMT in mice. In consistency with decreased calbindin levels, degenerated neurons stained by FJB appeared within 2 days after the administration and disappeared by 90 days later in the granule cell layer (Fig. 2). However, no FJB-positive signals were detected in the CA1 of hippocampal slices obtained at 1–90 days after the administration of TMT at all (data not shown). These results suggest the possible delayed and sustainable expression of both $GABA_BR1$ and $GABA_BR2$ subunits by cells other than calbindin-positive neurons and GFAP-positive astrocytes in the DG at 90 days after a single injection of TMT.

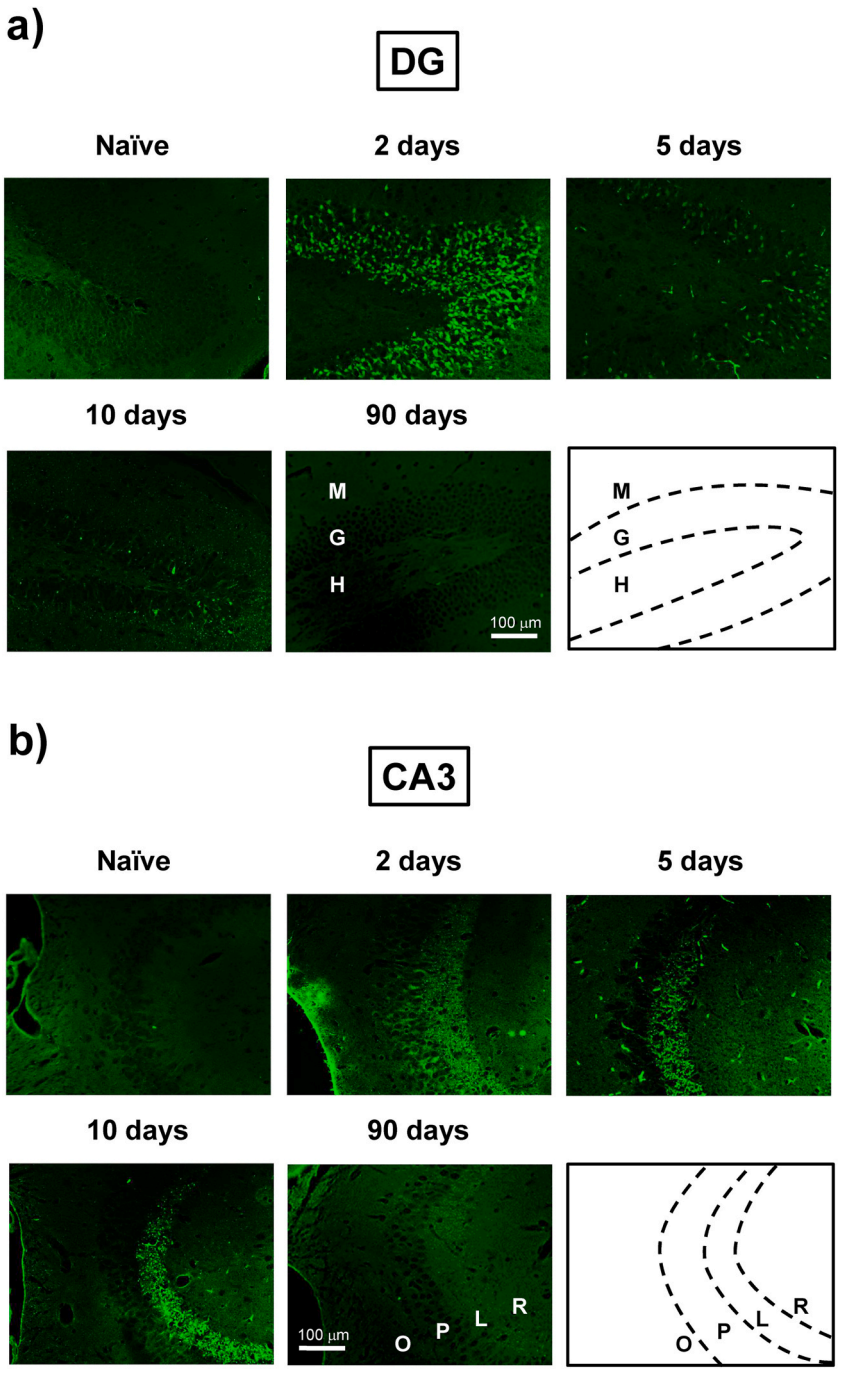

Fig. 2. Fluoro-Jade B signals in hippocampal sections at different days after TMT. Animals were given TMT (2.8 mg/kg, i.p.) and then fixed for sagittal sections on different days up to 90 days after the administration. Sections were stained with FJB and photos were taken around areas of the a) DG and b) CA3 region. There was no FJB signals in sections obtained on 90 days after the administration (Scale bar: 100 μm). M: molecular layer, G: granule cell layer, H: hilus, O: stratum oriens, P: pyramidal cell layer, L: stratum lucidum, R: stratum radiatum.

An attempt was thus made to further confirm the expression of $GABA_BR2$ subunit in cells other than astroglia at a late phase of TMT neurotoxicity in the murine DG. As $GABA_BR1a$ and $GABA_BR1b$ subunits were not detected in cells immunoreactive to GFAP, MAP-II or Tubulin-III in the DG at 30 days after the administration [6], we decided to focus on the evaluation of possible co-localization of only $GABA_BR2$ subunit with GFAP-positive cells in the DG in this study. In hippocampal sections obtained at 30 days after the administration (Fig. 3, upper panels), $GABA_BR2$ subunit was found in most cells expressing GFAP as shown previously [6]. In hippocampal sections obtained at 90 days after the administration of TMT (Fig. 3, lower panels), double positive cells for both $GABA_BR2$ subunit and GFAP were also detected in the hilus area, granule and molecular layers as seen in those at 30 days after the administration. Although $GABA_BR2$-positive signals were highly detected in cells with a fibrous shape without GFAP-positive signals in the granule layer on sections at 90 days after the administration, in contrast, GFAP-positive signals were solely seen in cells with neuron-like morphology.

4. Discussion

Several independent lines of evidence indicate the constitutive and inducible expression of both $GABA_BR$ subunits by microglial cells derived from macrophages in the brain. For example, $GABA_BR1a$, $GABA_BR1b$ and $GABA_BR2$ subunits were all found in astrocytes and activated microglia besides neurons, but not in myelin-forming oligodendrocytes, in the rat CNS [9]. A $GABA_BR$ agonist is shown to attenuate the release of interleukins by lipopolysaccharide in cultured microglia expressing both $GABA_BR$ subunits [10]. In transgenic mice expressing a fluorescent Ca^{2+} indicator specifically in microglia, electrical stimulation of the Schaffer collateral fibers led to marked Ca^{2+} responses in astrocytes followed by subsequent similar responses in microglia afterward in the hippocampus in a tetrodotoxin-sensitive manner during early postnatal stages [11]. These microglial Ca^{2+} responses were abolished not only by astrocytic glutamate uptake blockade but also by microglial $GABA_BR$ antagonism [11]. The possibility that both $GABA_BR$ subunits would be slowly expressed by microglial cells induced after the neurotoxic burden by TMT later than in astrocytes as a neuroinflammatory response in the brain is not ruled out. Indeed, astrocytes are shown to synthesize and secrete GABA to communicate with adjacent microglial cells through both $GABA_AR$ and $GABA_BR$ [12].

To our knowledge, this is the first direct demonstration of long-lasting and sustainable increases in both $GABA_BR$ subunit protein levels in the murine hippocampal DG up to 90 days after a single injection of TMT. Co-localization of $GABA_BR$ subunits with several microglial markers, however, should be evaluated in hippocampal preparations obtained at 90 days after the administration in future studies. The reason and mechanism for the current delayed and long-lasting upregulation of each $GABA_BR$ subunit in the DG after TMT intoxication are not clarified so far. Moreover, the inconsistent co-localization profile between $GABA_BR1$ and $GABA_BR2$ subunits [6] gives rise to an idea that each subunit would be endowed to orchestrate a functional homodimer required for modulation of adenylyl cyclase as a member of G-protein-coupled receptor family in the brain [13]. The absence of both $GABA_BR1a$ and $GABA_BR1b$ subunits from cells stained with $GABA_BR2$ subunit and GFAP in the DG at 30 days after the administration of TMT [6] discouraged us to examine the possible co-localization of each $GABA_BR1$ subunit with GFAP-positive cells in the DG at 90 days after the administration in this study. It is thus conceivable that GABA could play a role other than a neurotransmitter through a mechanism relevant to constitutive

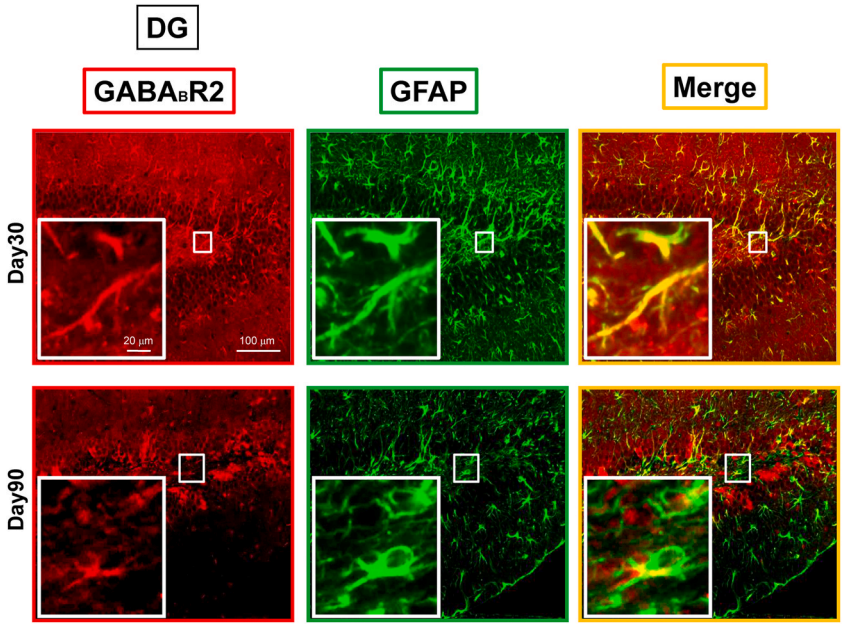

Fig. 3. Distribution of $GABA_BR2$ subunit in DG at 30 and 90 days after TMT. Animals were given TMT (2.8 mg/kg, i.p.) and then fixed on 30 or 90 days after the administration, which were subjected to immunostaining for $GABA_BR2$ (red) together with GFAP (green). Photos were taken around the area of the DG (Scale bar: 100 μm). Enlarged inset photos were obtained from an area of the dentate hilus (Scale bar: 20 μm).

and inducible expression of $GABA_BR$ subunits by cells of diverse phenotypes including neurons, astrocytes and microglia in response to a variety of endogenous and exogenous signal inputs in the brain.

Microglial activation after TMT was observed in the CA regions of rats and persisted for 2 months [14]. A possible microglial marker, endothelial monocyte-activating polypeptide-II expression was increased in the CA regions after TMT, peaking at 21 days and returning to baseline by 35 days [15]. In these reports, the response was not in the DG but in the CA regions, and furthermore, there is no mention of the regeneration process in the CA1 regions. As the effects of TMT differ between rodents, cares must be taken when applying it to humans. On the other hand, our mouse model can be said to be an excellent research model if used as a model for neurodegeneration and regeneration in the DG. In our preliminary study, protein expression of the microglial marker Iba1 and Iba1-positive cells significantly increased in the DG by day 5 after TMT, although these returned to baseline or disappeared by day 30 (data not shown). Therefore, $GABA_BR2$ is unlikely to be co-stained with Iba1-positive cells, so this study has not been carried out. Among the six types of microglial markers, Iba1-negative cells play a role in the survival and differentiation of neural stem cells in the lateral subventricular zone and in the migration of neuroblasts to the olfactory bulb through the rostral migratory stream [16]. Iba1-negative microglia is also present in the subgranular zone of the DG probably also to support neurogenesis in the DG [16]. Therefore, our next goal would be to investigate the possibility that $GABA_BR2$-positive cells are expressed in the minority but messianic microglia.

Activation of $GABA_BRs$ in neurons may contribute to resistance to neurofibrillary tangle formation [17]. $GABA_B$ agonists promoted proliferation and neuronal differentiation in neural progenitor cells that expressed both R1 and R2 subunits, but not in R1-null cells [18]. Decreased $GABA_BRs$ on macrophages leads to several changes observed in AD model mouse [19]. Those suggest that $GABA_B$ receptors in the hippocampus play an important role in suppressing in neurodegeneration and probably regeneration in the AD brain. Microglia are more abundantly observed in the DG in postmortem brains of AD patients [20]. There are two states of microglia: M1, which is associated with pro-inflammation, and M2, which is associated with anti-inflammation. Although the cytokines released by each state of cells are different, both are iba1 positive [21]. The iba1-negative microglia are cells involved in neurogenesis [16], and their role in AD brain remains largely unknown. The $GABA_BR2$-positive microglia may provide a clue to solving the identity of these cells. The temporary increase in microglia observed upon administration of TMT to rodents appears to be in response to the TMT-induced inflammation and ultimately to end inflammation [14,15]. In our study, TMT-induced damages in the mouse DG seemingly resolves by 30 days [6]. Day 90 should be a further regeneration stage post neuroinflammation. Homodimers of $GABA_BR2$ subunits are capable of localizing to membranes [22] and G-protein coupled receptors can exert their functions through membrane expression itself, and agonists can enhance their effects [23] so that those may thereby regulate adenylate cyclase activity. Therefore, it will be necessary to explore whether there are cells that express $GABA_BR2$ alone and what function it exerts.

5. Conclusion

In the TMT-treated mouse model in which transient neurodegeneration and regeneration can be observed, $GABA_BR2$-positive cells constitutively appeared in the DG, suggesting that they may direct the complete maturation of neural tissue during the regenerative process. The cells may be microglia which have not been fully understood but have properties supporting neuronal maturation. Although the results of this research will not directly lead to the treatment of AD, there is no doubt that elucidating this will provide a stepping stone to the development of regeneration and transplantation treatments for various neurodegenerative diseases, including AD.

Ethics statement

The protocol used here met the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University (K13–1, K14-1).

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Data availability statement

Our study is not deposited into a publicly available repository so data will be available on request.

CRediT authorship contribution statement

Yuki Onishi: Formal analysis, Investigation, Validation, Visualization, Writing – review & editing. **Yusuke Yamamura:** Formal analysis, Investigation, Visualization. **Misa Hosogi:** Formal analysis, Investigation, Visualization. **Hiroshi Higashi:** Formal analysis, Investigation, Visualization. **Kiyokazu Ogita:** Resources, Software, Conceptualization, Data curation, Methodology. **Toshihiko Kinjo:** Funding acquisition, Methodology, Software. **Kyosuke Uno:** Funding acquisition, Methodology, Software. **Yukio Yoneda:** Methodology, Software, Validation, Writing – review & editing. **Nobuyuki Kuramoto:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing –

review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29713.

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