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Opposing roles of glucocorticoid receptor and mineralocorticoid receptor in trimethyltin-induced cytotoxicity in the mouse hippocampus

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ABSTRACT

The organotin trimethyltin (TMT) is known to cause neuronal degeneration in the murine brain. Earlier studies indicate that TMT-induced neuronal degeneration is enhanced by adrenalectomy and prevented by exogenous glucocorticoid. The aim of this study was to investigate the regulation of TMT neuroxicity by corticosterone receptors including type I (mineralocorticoid receptor, MR) and type II (glucocorticoid receptor, GR) in adult mice. The systemic injection of TMT at the dose of 2.0 or 2.8 mg/kg produced a marked elevation in the level of plasma corticosterone that was both dose and time dependent. The MR agonist aldosterone had the ability to exacerbate TMT cytotoxicity in the dentate granule cell layer, whereas its antagonist spironolactone protected neurons from TMT cytotoxicity there. In contrast, the GR antagonist mifepristone exacerbated the TMT cytotoxicity. Taken together, our data suggest TMT cytotoxicity is oppositely regulated by GR and MR signals, being exacerbated by MR activation in adult mice.

As an adaptive response to numerous forms of systemic stress, the hypothalamic–pituitary–adrenocortical axis is activated. Activation of this axis promotes mainly the secretion of cortisol and cortisone in humans or corticosterone in rodents from the adrenal glands. In rodents, the hippocampus has 2 types of corticosteroid receptor, *i.e.*, type I (mineralocorticoid receptor, MR) and type II (glucocorticoid receptor, GR) [3].The MR has high affinity for corticosterone and is most densely localized in hippocampal and septal neurons. On the other hand, the GR show slow affinity for corticosterone and is ubiquitously distributed in the brain including in neurons in the hippocampus and hypothalamus and inglial cells. Both receptors complement each other in a way that allows modulation of the response of the limbic–hypothalamic–pituitary–adrenal (LHPA) axis. The MR is extensively occupied and activated at low corticosterone concentrations and may offer tonic inhibition of the LHPA axis most of the time [1,12]. When high concentrations of corticosterone are present, the MRs become saturated and the GRs become engaged. The dual action of these receptors in the hippocampus appears to be central for both basal modulation and stress regulation of the LHPS axis.

The organotin trimethyltin chloride (TMT) produces neuronal damage in both human and rodent central nervous systems. An acute treatment with TMT is known to increase temporally the concentration of plasma corticosterone in rats [4,17]. Abolishment of this TMT-induced increase in the corticosterone level by an inhibitor of corticosterone synthesis prevents TMT from damaging the hippocampal neurons and eliciting learning impairment and hyperactivity in rats [16]. Contrariwise, there exists an opposing report indicating that in adrenalectomized rats the depletion of circulating corticosterone facilitates TMT-induced damage to CA3 pyramidal cells of the hippocampus [5]. To date, however, there are no findings on the roles of MRs and GRs in TMT-induced neuronal damage in the murine brain. To this end, we investigated the effect of agonists and antagonists of MRs and GRs on neuronal injury in the TMT mouse model, a model well established in our previous studies [7,10,11,21,22]. The main goal of the study was to elucidate the roles played by MR and GR in TMT-induced neurotoxicity.

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. Adult male Std-ddY mice, weighing 30–35 g and 5–6 weeks of age, were intraperitoneally injected with TMT (2.8 or 2.0 mg/kg, Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in

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phosphate-buffered saline, and then returned to their home cages until the time of decapitation.

For histological assessments, mice were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.) and perfused *via* the heart with saline, followed by 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Their brains were quickly removed and further fixed with the same fixative solution at 4 °C overnight. Post-fixed brains were embedded in paraffin and then cut as coronal sections of 3-μm thickness with a microtome. Some sections so obtained were subjected to immunostaining analysis using a rabbit antibody against single-stranded DNA (ssDNA, Dako Japan Co. Ltd., Kyoto, Japan) as described previously [7,21]. Fluoro-Jade-B staining of brain sections was used to identify degenerating neurons [14]. Briefly, the coronal sections were placed on a shaker in 0.06% potassium permanganate for 10 min and washed in distilled water before immersion in a 0.006% Fluoro-Jade B (Millipore, Billerica, MA, USA) for 20 min. Stained sections were viewed with an Olympus U-LH100HG fluorescence microscope, and the number of cells was counted by microscopic observation.

For immunoblot analysis, hippocampal tissue lysates were prepared by homogenizing the tissue in 1 mL of 10 mM Tris–HCl buffer (pH 7.5) containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, phosphatase inhibitors (10 mM sodium β-glycerophosphate and 1 mM sodium orthovanadate), and 1 μg/mL each of protease inhibitors [(p-amidinophenyl)methanesulfonyl fluoride, benzamidine, leupeptin, and antipain]. Protein concentrations were measured by using the Protein Assay Rapid kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Immunoblot assays were carried out by using primary antibodies against GR, MR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology Inc., Santa Cruz., CA, USA) as described previously [15].

All data were expressed as the mean ± S.E.M., and the statistical significance was determined by the two-tailed Student *t*-test or the one-way ANOVA with Bonferroni/Dunnett *post hoc* test.

We initially determined the level of corticosterone in the plasma prepared on days 1 and 2 post-treatment with TMT at the dose of 2.0 (low dose) or 2.8 mg/kg (high dose). Expectedly, the level of plasma corticosterone was dramatically elevated on days 1 and 2 post-TMT treatment with the high dose (Fig. 1a). When examined on day 2 post-treatment, the elevation of the plasma corticosterone level was TMT dose dependent (plasma corticosterone [ng/mL, $n = 5$]: vehicle, 14.7 ± 0.3; TMT [2.0 mg/kg], 28.1 ± 0.5 [$P < 0.05$]; TMT [2.8 mg/kg], 116 ± 5 [$P < 0.001$]; Fig. 1b), being parallel to the TMT-induced cytotoxicity in the dentate granule cell layer (number of ssDNA-positive cells/mm^2 [$n = 4$]: vehicle, not detectable; TMT [2.0 mg/kg], 156 ± 26; TMT [2.8 mg/kg], 853 ± 51). The level of both corticosterone receptors including the GR and MR had no significant change at least on day 2 post-treatment with TMT at 2.0 mg/kg (Fig. 2).

Fluoro-Jade B staining revealed that the single systemic injection of TMT at 2.0 mg/kg produced degenerating neurons in the dentate granule cell layer on day 2 post-treatment (Fig. 3a). We next examined the effect of aldosterone as an agonist of MRs on the TMT-induced cytotoxicity (Fig. 3b and c). Co-treatment of normal mice with TMT and aldosterone markedly increased the number of ssDNA-positive cells in the dentate granule cell layer on day 2 post-treatment, compared to that of animals treated TMT alone. In adrenalectomized mice, aldosterone was also effective in enhancing the number of ssDNA-positive cells in the dentate gyrus of TMT-treated mice under the same experimental schedule (number of ssDNA-positive cells/mm^2 [$n = 6$]: TMT alone, 214 ± 21; TMT/aldosterone, 296 ± 29 [$P < 0.05$]).

Our previous report showed that mifepristone (GR antagonist) was effective in exacerbating TMT neurotoxicity in the dentate gyrus [15]. To evaluate regulation by endogenous corticosterone

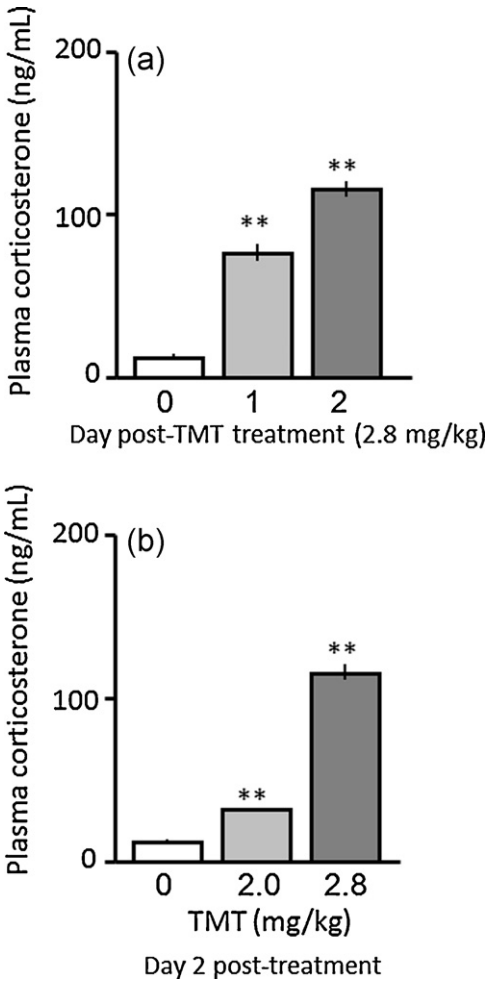

Fig. 1. Effect of TMT on the level of plasma corticosterone. Animals were given either saline or TMT (2.0 or 2.8 mg/kg, i.p.) and then decapitated on day 1 or 2 post-treatment for collection of blood samples from the heart. After the blood samples had been centrifuged at 1000 × *g* for 15 min, the plasma was immediately taken for determination of corticosterone levels by using a corticosterone EIA kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.). Time course (a) and dose–response relationship (b) are shown. Values are the mean ± S.E.M. from 5 separate experiments.**$P < 0.01$, significantly different from control value obtained for saline-treated animals (*x*-axis = 0).

via MRs and GRs in TMT neurotoxicity, we determined the effect of spironolactone (MR antagonist) and mifepristone on the TMT-induced appearance of ssDNA-positive cells in the dentate granule cell layer (Fig. 3b and c). Spironolactone was effective in reducing this number of ssDNA-positive cells in the dentate granule cell layer of TMT-treated animals. Under the same experimental conditions, mifepristone increased the number of ssDNA-positive cells there (number of ssDNA-positive cells/mm^2 [$n = 6$]: TMT alone, 158 ± 25; TMT/mifepristone, 237 ± 20 [$P < 0.05$]).

Corticosterone activates MRs and GRs, with the former receptor binding corticosterone with an affinity 10-fold higher than the latter one [3]. Thus, at low basal levels corticosterone predominantly occupies MRs, whereas GRs can be activated additional to MRs only when corticosterone levels are high, *i.e.*, at the circadian peak and during stress. In the present study, we demonstrated that *in vivo* treatment with TMT at 2.0 mg/kg elevated the plasma level of corticosterone to one at least 3 times higher than that in the untreated animals. In addition, the MR and GR contents showed no significant change following TMT treatment at 2.0 mg/kg. These findings support the proposition that *in vivo* treatment with TMT at 2.0 mg/kg activates both MR and GR in the hippocampus. Since corticosterone

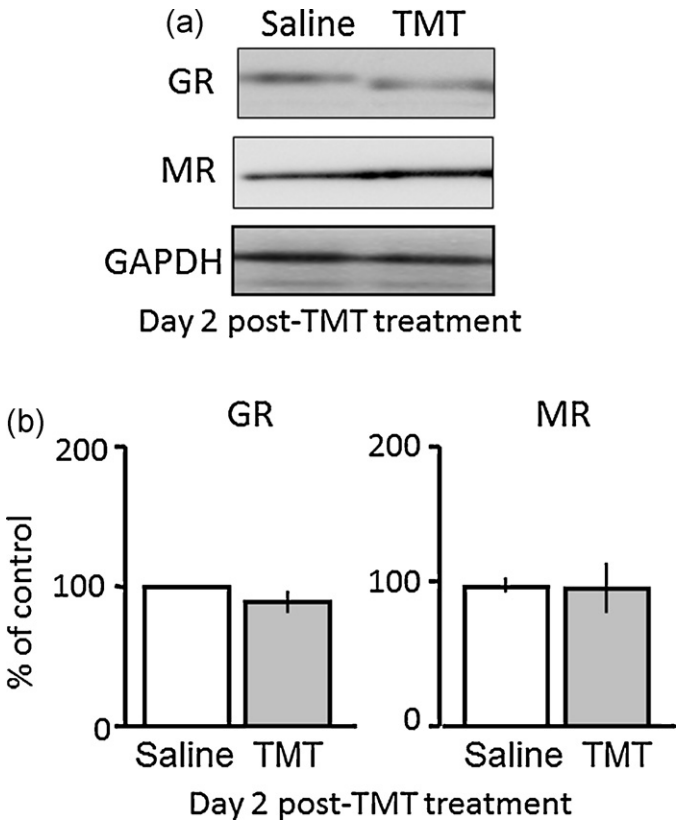

Fig. 2. Effect of TMT on the levels of GR and MR in the hippocampus. Animals were given either saline or TMT (2.0 mg/kg, i.p.) and then decapitated on day 2 post-treatment for preparation of lysates of the hippocampus. The tissue lysates were subjected to immunoblot analysis for determination of GR and MR levels as well as that of GAPDH level as an internal control. (a) Typical immunoblots. (b) Quantitative data of GR (left panel) and MR (right panel). Values are the mean ± S.E.M. from 6 separate experiments. $^{**}P < 0.01$, significantly different from the control value obtained for saline-treated animals (x-axis = 0).

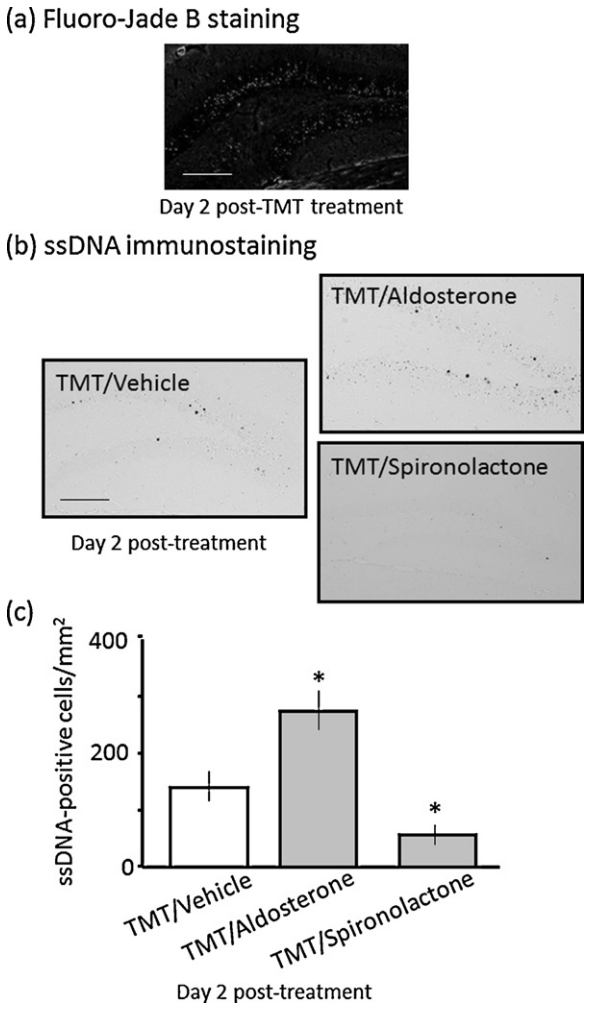

Fig. 3. Effect of aldosterone and spironolactone on TMT-cytotoxicity in the dentate granule cell layer. Animals were given either vehicle, aldosterone (0.1 mg/kg, s.c., Sigma–Aldrich Co., St. Louis, MO, USA) or spironolactone (100 mg/kg, s.c., Sigma–Aldrich Co., St. Louis, MO, USA) along with TMT (2.0 mg/kg, i.p.) and then treated at a 12-h interval with either vehicle, aldosterone or spironolactone at the same dose until day 2 post-TMT treatment. After having been fixed with paraformaldehyde, their brains were then removed and embedded in paraffin. Subsequently, coronal sections (Bregma 2.8–3.0 mm) of the hippocampus were prepared from the paraffin-embedded brains and then stained with anti-ssDNA antibody. (a) A typical fluorescence micrograph of Fluoro-Jade B staining in the dentate gyrus on day 2 post-treatment with TMT alone. Fluoro-Jade B-positive cells (degenerating neurons) are shown as white dots. (b) Typical light micrographs of ssDNA immunostaining in the dentate gyrus on day 2 post-treatment. ssDNA-positive cells are shown as black dots. Scale bar = 200 μm. (c) Quantitative data of the density of ssDNA-positive cells is expressed as the mean ± S.E.M. per mm^2 of the dentate gyrus from 6 separate experiments. $^{*}P < 0.05$, significantly different from control value obtained for animals treated with TMT and vehicle (TMT/vehicle).

receptors is known to exist in both glial cells and neurons in the hippocampus [16], TMT is capable of activating GR and MR in both glial cells and neurons through elevation of endogenous corticosterone.

Under these conditions in the current study, the essential importance of our findings is that the agonist or antagonist for MRs had the respective ability to exacerbate or ameliorate TMT neurotoxicity in the dentate gyrus. Thus, in this study we show for the first time that MR activation contributes to TMT-induced neuronal damage in the dentate gyrus. Because TMT was capable of degenerating dentate neurons in adrenalectomized mice, in which the level of plasma corticosterone is negligible [15], it is most likely that MR activation positively modulates, but is not essential, TMT neurotoxicity in the dentate gyrus. The finding that the blockage of GRs by mifepristone exacerbated TMT neurotoxicity supports a previous proposition that GR activation prevents TMT neurotoxicity [15].

Considerable evidence suggests that under the physiological conditions, MR expression is associated with a neuroprotective phenotype, whereas GR activation has been implicated in the induction of an endangered neural phenotype and these opposite actions are most evident in the hippocampus, where these receptors predominate [13]. Under neuropathological conditions, it was earlier demonstrated that forebrain-specific MR-overexpressing transgenic mice have significantly reduced neuronal death following transient cerebral global ischemia compared to wild-type littermates [8]. Conversely, there is a recent report showing that direct exposure to aldosterone produces cell damage in cultures of spinal ganglion cells [9]. Further evidence of a GR-dependent neuroprotective action comes from the previous findings that dexamethasone has the ability to prevent TMT neurotoxicity in the hippocampus of adrenalectomized mice [15]. Thus, MR and GR play opposing roles in TMT-mediated cell degeneration. Furthermore, we conclude that endogenous corticosterone can function as a multi-regulating factor in TMT neurotoxicity.Accumulating evidence suggests that glucocorticoid negatively interacts with nuclear transcription factor including nuclear factor-κB and activator protein-1 in various cells [2,18]. Glucocorticoids are known to alleviate lipopolysaccharide-induced brain injury by inhibiting nuclear factor-κB activation [19]. In addition to nuclear factor-κB, inhibition of activator protein-1 by interaction with GR protects light-induced apoptosis in retinal photoreceptor cells [20]. In rat renal fibroblasts, on the other hand, aldosteroneis shown to enhance the expression of nuclear factor-κB and activator potein-1

with being prevented by spironolactone [6]. Therefore, it is likely that GR and MR have an opposite role in regulating activity of nuclear factor-κB and activator potein-1. In the present study, the opposite role of GR and MR in TMT-mediated cytotoxicity could be due to interaction with these transcription factors. Indeed, the acute treatment with TMT activates activator potein-1 in the murine hippocampus [10]. However, it remains for future studies to elucidate the molecular mechanism underlying the involvement of MRs in TMT-mediated neurotoxicity.

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