



## ARTICLE

# T-448, a specific inhibitor of LSD1 enzyme activity, improves learning function without causing thrombocytopenia in mice

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Dysregulation of histone H3 lysine 4 (H3K4) methylation has been implicated in the pathogenesis of several neurodevelopmental disorders. Targeting lysine-specific demethylase 1 (LSD1), an H3K4 demethylase, is therefore a promising approach to treat these disorders. However, LSD1 forms complexes with cofactors including growth factor independent 1B (GFI1B), a critical regulator of hematopoietic differentiation. Known tranylcypromine-based irreversible LSD1 inhibitors bind to coenzyme flavin adenine dinucleotide (FAD) and disrupt the LSD1-GFI1B complex, which is associated with hematotoxicity such as thrombocytopenia, representing a major hurdle in the development of LSD1 inhibitors as therapeutic agents. To discover LSD1 inhibitors with potent epigenetic modulation and lower risk of hematotoxicity, we screened small molecules that enhance H3K4 methylation by the inhibition of LSD1 enzyme activity in primary cultured rat neurons but have little impact on LSD1-GFI1B complex in human TF-1a erythroblasts. Here we report the discovery of a specific inhibitor of LSD1 enzyme activity, T-448 (3-((1*S*,2*R*)-2-(cyclobutylamino) cyclopropyl)-*N*-(5-methyl-1,3,4-thiadiazol-2-yl)benzamide fumarate). T-448 has minimal impact on the LSD1-GFI1B complex and a superior hematological safety profile in mice via the generation of a compact formyl-FAD adduct. T-448 increased brain H3K4 methylation and partially restored learning function in mice with NMDA receptor hypofunction. T-448-type LSD1 inhibitors with improved safety profiles may provide unique therapeutic approaches for central nervous system disorders associated with epigenetic dysregulation.

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## INTRODUCTION

Epigenetic control of gene transcription by histone H3 lysine 4 (H3K4) methylation is critically involved in neural functions such as learning and memory through the regulation of synaptic plasticity [1–3]. Upregulation of H3K4 methylation levels allows active transcription of genes via recruitment of chromatin remodeling factors. Reduced methylation of H3K4, and the consequent reduction of gene transcription, have been implicated in the pathogenesis of various psychiatric and neurological disorders, such as schizophrenia [4, 5], autism spectrum disorders (ASD) [6, 7], intellectual disability disorders [8], and Huntington's disease [9]. Therefore, treatments which restore normal levels of H3K4 methylation may be a promising approach to treat disorders associated with this type of epigenetic dysfunction.

Lysine-specific demethylase 1 (LSD1, also known as KDM1A) is an enzyme that decreases methylation levels, predominantly at H3K4 [10]. Interestingly, LSD1 only demethylates histone H3 lysine 9 (H3K9) when the molecule forms a complex with an androgen receptor [11]. Non-histone proteins such as DNMT1 [12] are also known LSD1 substrates. At H3K4, LSD1 catalyzes the oxidative removal of methyl groups from mono- and dimethylated H3K4 by reducing flavin adenine dinucleotide (FAD)

into FADH<sub>2</sub> [13–16]. The principal target site of known irreversible LSD1 inhibitors is FAD, located within the active site cavity of LSD1 [17]. This appears to be the most promising approach to discover LSD1 inhibitors, as no allosteric LSD1 inhibitors have been identified.

LSD1 interacts with multiple cofactor proteins such as growth factor independent 1B (GFI1B), a Snail/Gfi1 (SNAG) domain family protein in the vicinity of FAD binding site [18, 19]. GFI1B regulates the hematopoietic differentiation of erythroid and megakaryocytic cells by repressing the transcription of most of its target genes, including growth factor independent 1 (*Gfi1*) [20, 21]. Depletion of LSD1 by knockdown in mice derepressed the expression of the genes controlled by GFI1B [19, 22] and attenuated the production of peripheral blood cells by perturbing hematopoietic differentiation. Moreover, known irreversible LSD1 inhibitors with a tranylcypromine structure that produce FAD-adducts have shown in vivo hematological toxicities, such as thrombocytopenia [23–25], probably through disruption of the interaction between LSD1 and cofactors [26, 27]. Reversible inhibitors have not been reported to form FAD-adducts. Hematological toxicity caused by disruption of the LSD1-complex may pose a major hurdle in the development of LSD1 inhibitors as therapeutic agents.

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Here, we report the discovery of a novel selective LSD1 inhibitor, 3-((1*S*,2*R*)-2-(cyclobutylamino)cyclopropyl)-*N*-(5-methyl-1,3,4-thiadiazol-2-yl)benzamide fumarate (T-448) using an original screening strategy. T-448 irreversibly inhibited the enzyme activity of LSD1 through the generation of a compact formyl-FAD adduct. Formation of the formyl-FAD adduct had minimal impact on the interaction between LSD1 and GF11B and as expected, T-448 at the effective dose did not show hematological toxicity in mice. In mice with *N*-methyl-D-aspartate receptor (NMDAR) hypofunction, a preclinical model of schizophrenia and ASD [28–30], T-448 enhanced the levels of H3K4 methylation, increased mRNA expression of neural plasticity-related genes including brain derived neurotrophic factor (*Bdnf*), and ameliorated learning dysfunction as measured in the water Y-maze test. The evidence we provide here suggests that T-448-type LSD1 inhibitors may provide a novel therapeutic opportunity for central nervous system (CNS) diseases associated with epigenetic dysregulation.

## MATERIALS AND METHODS

See supplemental information for detailed methods. The methods of statistical analysis and criteria of significance are indicated in each Figure legend.

### Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Shonan Research Center, Takeda Pharmaceutical Company Limited. Male Jcl:ICR mice were obtained from CLEA Japan, Inc. Primary neuronal culture was prepared from Crl:CD(SD) rats at embryonic day 19 purchased from Charles River Laboratories Japan, Inc. [31].

### TF-1a culture

TF-1a was purchased from the American Tissue Culture Collection. Cell culture, viability assay, and immunoprecipitation were performed as previously described [23] with minor modifications.

### H3K4me2 ChIP studies

ChIP was performed by using ChIP-IT Express Enzymatic (Active Motif, #53009) or OneDay ChIP Kit (Nippon Gene, #313-80461) and H3K4me2 antibody (Millipore, #07-030) with a slight modification to the manufacturer's instruction. Sequencing was performed by Takara Bio Inc. (HiSeq2000).

### mRNA expression studies

mRNA expression was quantified by using FastLane kit (QIAGEN) or RNeasy Plus Kit (QIAGEN) and TaqMan reagents (Eurogentec) following the manufacturer's instruction. Microarray study was performed by Takara Bio Inc. (SurePrint G3 Rat Gene Expression 8 × 60K).

### SPR analysis

Experiments for binding to recombinant human LSD1 protein were carried out using Biacore 4000 instrument equipped with Sensor chip CM5 (GE Healthcare).

### Structural analyses

Crystals were obtained using the sitting-drop vapor-diffusion method. Diffraction data for each complex crystal were collected at 100 K at the Advanced Light Source beamline 5.0.3 or 5.0.2 (Berkeley). ESI-triple quadrupole mass spectrometry was used for the analysis of FAD-adducts.

### Ex vivo LSD1 enzyme assay

LSD1 protein in mouse tissue homogenates was immunoprecipitated by using anti-LSD1 antibody (Cell Signaling Technology, #2139) and Protein G Sepharose 4FF, according to the

manufacturer's instructions. Histone demethylase activity in immunoprecipitates was measured by HTRF™ detection system.

### Cognition test

Learning function was assessed by water Y-maze test using NR1-hypo (*Nr1<sup>neo-/-</sup>*) mice and their wild-type littermates [28, 29].

## RESULTS

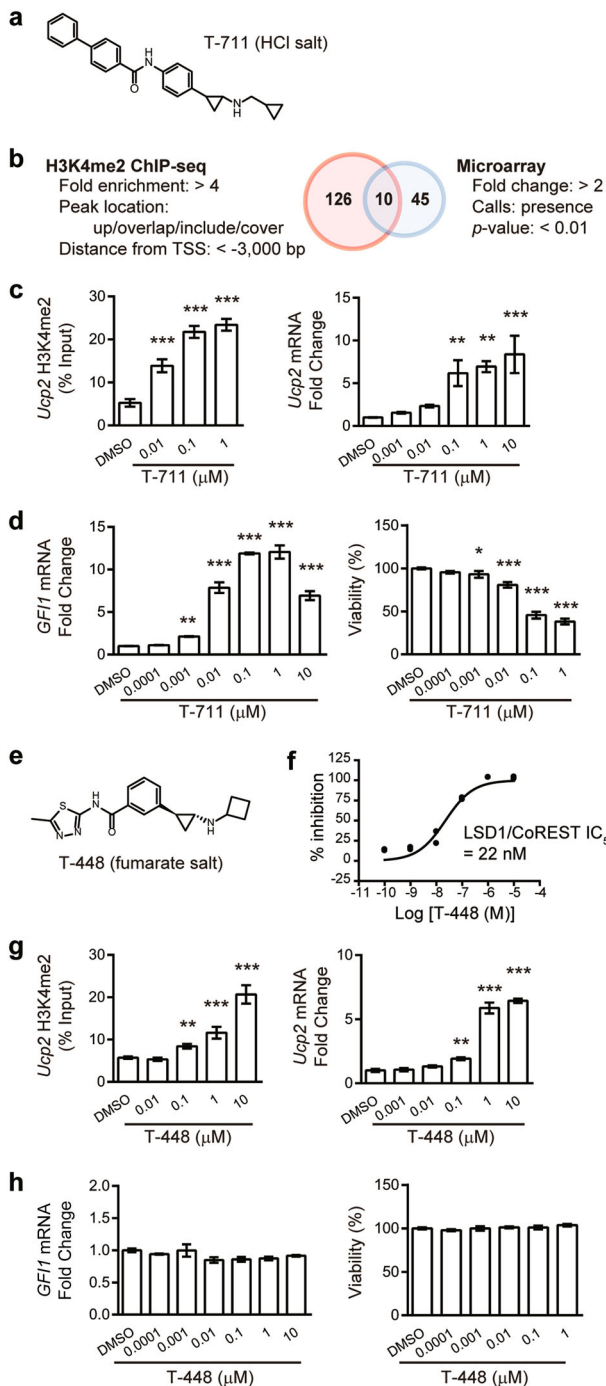
### Discovery of a novel LSD1 inhibitor, T-448

To discover LSD1 inhibitors with potent epigenetic modulation and lower risk of hematological toxicity, we set the following three criteria for the selection of lead compounds: (1) selective inhibition of the enzyme activity of purified human recombinant LSD1, (2) increase in H3K4 methylation levels and the consequent induction of gene transcription in primary cultured rat neurons, and (3) minimal impact on the transcription of *GF11* gene in human TF-1a erythroblast cell line [19, 22]. We also assessed the dissociation of the LSD1-GF11B complex as a representative mechanism that controls *GF11* mRNA expression in TF-1a cells [20, 21].

Chromatin immunoprecipitation (ChIP) is a method to quantitatively evaluate H3K4 methylation levels at specific genomic regions; however, the high-throughput screening of drugs using ChIP was technically difficult owing to the required amounts of primary cultured neurons and the complicated procedures involved. Therefore, we decided to use the induction of surrogate marker genes for the initial search. Surrogate marker genes were identified using T-711 (Fig. 1a), a racemic irreversible-type LSD1 inhibitor with specificity constant  $k_{\text{inact}}/K_i$  of  $3.8 \times 10^5 \pm 1.9 \times 10^4$  ( $\text{s}^{-1} \text{M}^{-1}$ ) (Fig. S1) [32]. The genes with increased levels of H3K4 methylation and mRNA expression after treatment with T-711 in primary cultured rat neurons represented candidates for surrogate marker genes. The effects of T-711 (1  $\mu\text{M}$ ) on H3K4 di-methylation (H3K4me2) in primary cultured rat neurons were studied by ChIP-sequencing (ChIP-seq) using an antibody against H3K4me2 in a genome-wide manner. The sequence reads were mapped to rat genomes (rn4), and H3K4me2 peaks were called by model-based analysis of ChIP-seq (MACS) algorithm. Among them, peaks located within genes or promoter regions were annotated to the gene with the nearest transcription start sites (TSSs). As a result, we identified 136 genes with more than 4-fold increases in H3K4me2 levels after treatment with T-711 (Fig. 1b). The changes in mRNA expression induced by T-711 (1  $\mu\text{M}$ ) in primary cultured rat neurons were analyzed through a microarray study; 55 genes showed more than 2-fold increased expression (Fig. 1b). Among the 10 genes identified by both H3K4me2 ChIP-seq and microarray analyses, *Ucp2* mRNA showed the most robust fold induction (Table S1). H3K4me2 ChIP-quantitative PCR (qPCR) and RT-qPCR analyses revealed that T-711 significantly increased the H3K4me2 level at the upstream region (−239 base pairs from the TSS) of the *Ucp2* gene, and increased mRNA expression of the *Ucp2* gene in a concentration-dependent manner in primary cultured rat neurons (Fig. 1c). Therefore, we decided to utilize the *Ucp2* mRNA levels in primary cultured rat neurons as a surrogate marker to guide discovery of LSD1 inhibitors.

GF11 regulates hematopoietic differentiation, and *GF11* mRNA transcription is controlled by an LSD1-complex including GF11B in hematopoietic lineage cells [19, 21, 22]. Therefore, we decided to use the induction of *GF11* mRNA expression in TF-1a erythroblast cell lines as a screening index of hematological toxicity. RT-qPCR analysis confirmed that T-711 significantly increased the *GF11* mRNA expression in a concentration-dependent manner in TF-1a cells (Fig. 1d). T-711 reduced the viability of TF-1a cells at the concentrations where it induced the *GF11* mRNA expression (Fig. 1d).

We initially screened our tranylcyproline-focused compound library (663 compounds) and discovered 440 compounds with inhibitory activity against human recombinant LSD1 (<100 nM



**Fig. 1** T-448 inhibited enzyme activity of LSD1 and increased H3K4me2 in neurons but did not impact the *GF11* mRNA levels in TF-1a erythroblast cells. **a** Chemical structure of T-711. **b** Venn diagram showing the numbers of genes with increased H3K4me2 and mRNA expression by T-711 treatment (1  $\mu$ M) for 3 days in primary cultured rat neurons. Detailed information of overlapped 10 genes is shown in Table S1. **c** Effects of 3-day treatment with T-711 on *Ucp2* gene H3K4me2 and *Ucp2* mRNA levels in primary cultured rat neurons. Graphs indicate mean  $\pm$  SEM.  $n = 4$  (DMSO group in H3K4me2 assay) or 3 (other groups). One-tailed parametric Williams' test vs DMSO-treatment group,  $**p < 0.005$ ,  $***p < 0.0005$ . **d** Effects of T-711 on *GF11* mRNA levels (1-day treatment) and viability (3-day treatment) in TF-1a erythroblast cells. Graphs indicate mean  $\pm$  SEM.  $n = 18$  (DMSO group in *GF11* assay),  $n = 15$  (DMSO group in viability assay), or 3 (compound treatment groups). One-tailed (*GF11* assay) and two-tailed (viability assay) parametric Williams' test vs DMSO-treatment group,  $*p < 0.025$ ,  $**p < 0.005$ ,  $***p < 0.0005$ . **e** Chemical structure of T-448. **f** Inhibition of LSD1 enzyme activity by T-448. The  $IC_{50}$  value was determined as 22 nM (95% confidence interval (CI): 13–40 nM).  $n = 2$ . **g** Effects of 3-day treatment with T-448 on *Ucp2* gene H3K4me2 and *Ucp2* mRNA levels in primary cultured rat neurons. Graphs indicate mean  $\pm$  SEM.  $n = 3$ . One-tailed parametric Williams' test vs DMSO-treatment group,  $**p < 0.005$ ,  $***p < 0.0005$ . **h** Effects of T-448 on *GF11* mRNA levels (1-day treatment) and viability (3-day treatment) in TF-1a erythroblast cells. Graphs indicate mean  $\pm$  SEM.  $n = 18$  (DMSO group in *GF11* assay),  $n = 15$  (DMSO group in viability assay), or 3 (compound treatment groups). No significance by one-tailed (*GF11* assay) and two-tailed (viability assay) parametric Williams' test vs DMSO-treatment group

LSD1 (Fig. S2) [33]. T-448 at 0.1  $\mu$ M or higher enhanced both the H3K4me2 level at the upstream region of *Ucp2* gene and the mRNA expression level of *Ucp2* gene in primary cultured rat neurons (Fig. 1g). On the other hand, T-448 at up to 10  $\mu$ M did not increase *GF11* mRNA expression and, at up to 1  $\mu$ M, did not affect the viability of TF-1a cells (Fig. 1h).

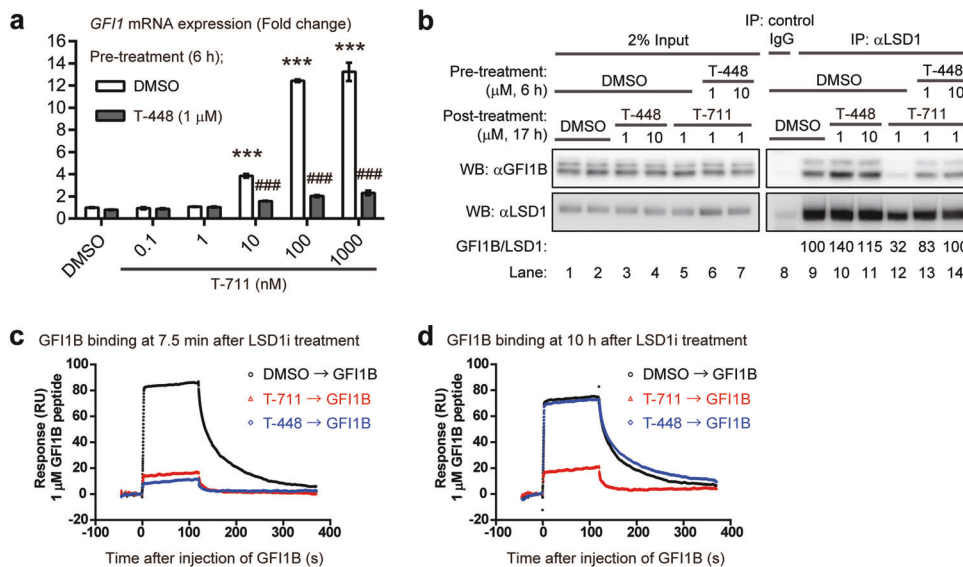
T-448 inhibits LSD1 enzyme activity with minimal impact on LSD1-GFI1B complex

To investigate the mechanism of action underlying the lack of *GF11* mRNA induction by T-448, we assessed the effects of T-448 on the T-711-induced *GF11* mRNA expression in TF-1a cells. TF-1a cells were pre-incubated with DMSO or T-448 (1  $\mu$ M) for 6 h, and then stimulated with various concentrations of T-711 for 24 h. T-448 pre-treatment significantly weakened the T-711-induced *GF11* mRNA expression (Fig. 2a). Thus, T-711 and T-448 may share the same binding site on LSD1.

To understand the impact of T-448 on the LSD1-complex, we evaluated its effects on the interaction between LSD1 and GFI1B in TF-1a cells by immunoprecipitation (IP) analysis, using T-711 as a control. As expected, T-711 robustly reduced the interaction between LSD1 and GFI1B (Fig. 2b, lanes 9, 12, and Fig. S3), while T-448 (1 and 10  $\mu$ M) did not disrupt the interaction (Fig. 2b, lanes 9–11). Next, TF-1a cells were pre-incubated with DMSO or T-448 (1 and 10  $\mu$ M) for 6 h, and then treated with T-711 (1  $\mu$ M) for 17 h. As a result, T-448 pre-treatment inhibited the T-711-induced dissociation between LSD1 and GFI1B (Fig. 2b, lanes 12–14). These data further supported the idea that both compounds occupy the same binding site on LSD1, and that T-448 has a lower effect on the interaction between LSD1 and GFI1B than T-711 in TF-1a cells.

The effects of LSD1 inhibitors on the binding between LSD1 and GFI1B were also investigated by surface plasmon resonance (SPR) biosensing assay (Fig. S4). Recombinant human LSD1 was immobilized on a sensor chip, and was then treated with T-711 (10  $\mu$ M) or T-448 (10  $\mu$ M). At both 7.5 min and 10 h after T-711 treatment, the binding of GFI1B (1  $\mu$ M) to the LSD1 was attenuated (Fig. 2c, d). T-448-treatment reduced the binding between GFI1B and LSD1 at 7.5 min after the reaction. At 10 h

$IC_{50}$ ). Next, we selected compounds that increased *Ucp2* mRNA expression in primary cultured rat neurons in a concentration-dependent manner (criteria:  $>2$ -fold change at 1  $\mu$ M) without affecting *GF11* mRNA expression in TF-1a cells (criteria:  $<2$ -fold change at 10  $\mu$ M). Then, two compounds were chosen because of the good selectivity against monoamine oxidase (MAO) A and B inhibition and their drug-likeness. Finally, T-448 (Fig. 1e) was selected owing to its better brain penetration profile. T-448 inhibited H3K4 demethylase activity of human recombinant LSD1 with an  $IC_{50}$  value of 22 nM in an irreversible manner:  $k_{inact}/K_i$  value of  $1.7 \times 10^4 \pm 2.6 \times 10^3$  ( $s^{-1} M^{-1}$ ) (Fig. 1f and Fig. S1). T-448 showed more than 4,500-fold selectivity over MAOA/B: the FAD-dependent enzymes with high amino acid sequence homology to



**Fig. 2** T-448 showed minimal impact on the LSD1-GFI1B complex. **a** Effects of pre-treatment with T-448 on T-711-induced *GFI1* mRNA expression in TF-1a cells. Graphs indicate mean ± SEM.  $n = 6$  (DMSO-treatment group) or 3 (T-711 treatment group). Concentration-dependent effects of T-711 were statistically analyzed by one-tailed parametric Williams' test,  $***p < 0.0005$ . Effects of T-448 pre-treatment on the inhibition of T-711 (10–1,000 nM)-induced *GFI1* mRNA expression were statistically analyzed by Student's *t*-test with Bonferroni correction,  $###p < 0.00033$ .  $n = 3$ . **b** IP analysis of the interaction between LSD1 and GFI1B in TF-1a cells. GFI1B/LSD1 values are obtained from the densitometry data of GFI1B bands normalized by LSD1 bands (lane 9 = 100%). **c, d** SPR analysis to measure the effects of LSD1 inhibitors on the interaction between LSD1 and GFI1B, 7.5 min (**c**) and 10 h (**d**) after the compound treatment (10 μM) on immobilized LSD1

after treatment with T-448, the interaction between GFI1B and LSD1 was recovered to the DMSO-control level (Fig. 2c, d). These results suggested that, differently from T-711, dissociation of the interaction between LSD1 and GFI1B by T-448 is only transient.

Formation of a compact FAD-adduct by T-448 permits LSD1-GFI1B interaction

We next analyzed the structures of coenzyme FAD in recombinant human LSD1 after treatment with LSD1 inhibitors. X-ray crystal analysis revealed that T-367 (Fig. S5), the eutomer of T-711, generated a covalent adduct with FAD in the active site cavity of LSD1 (Fig. 3a, stick model in white). LC/MS analysis was used to determine the molecular weight of FAD or FAD-adducts in recombinant LSD1. The peak with  $m/z$  784 represented intact FAD. LC/MS analysis of FAD in LSD1 treated by T-367 allowed the detection of a peak with  $m/z$  1,113, along with the FAD peak, confirming the formation of a T-367-FAD adduct (Fig. 3b). The N-terminal SNAG domain of GFI1B binds to the active site cavity of LSD1 [19]. The binding structure of GFI1B to LSD1 was simulated from the co-crystal analysis of LSD1 with N-terminal SNAIL1 peptide (PDB ID: 2Y48) [18], because the first 6 residues at the N-terminal are identical among SNAIL1 and GFI1B (peptide sequence: PRSFLV). As shown in Fig. 3a, the superimposed structures indicated a steric hindrance between the T-367-FAD adduct and GFI1B peptide (stick model in yellow). These results suggested that the bulky FAD-adduct generated by T-367 disrupts the interaction between LSD1 and GFI1B.

Interestingly, T-448 produced a reduced form of FAD with a formyl group (Fig. 3c and Fig. S6). The formation of a covalent adduct to FAD supports the irreversible inhibition of LSD1 enzyme activity. LC/MS analysis confirmed that the T-448 treatment generated a peak with  $m/z$  814, which corresponded to the molecular weight of FAD with formyl group, and a peak with  $m/z$  784 (FAD) (Fig. 3d). This LC/MS study also allowed the detection of a peak with  $m/z$  1,059, which corresponded to the molecular weight of a full T-448-FAD adduct (Fig. 3d). The  $m/z$  814 peak count increased while the  $m/z$  1,059 peak count decreased in a time-dependent manner, suggesting that the full T-448-FAD

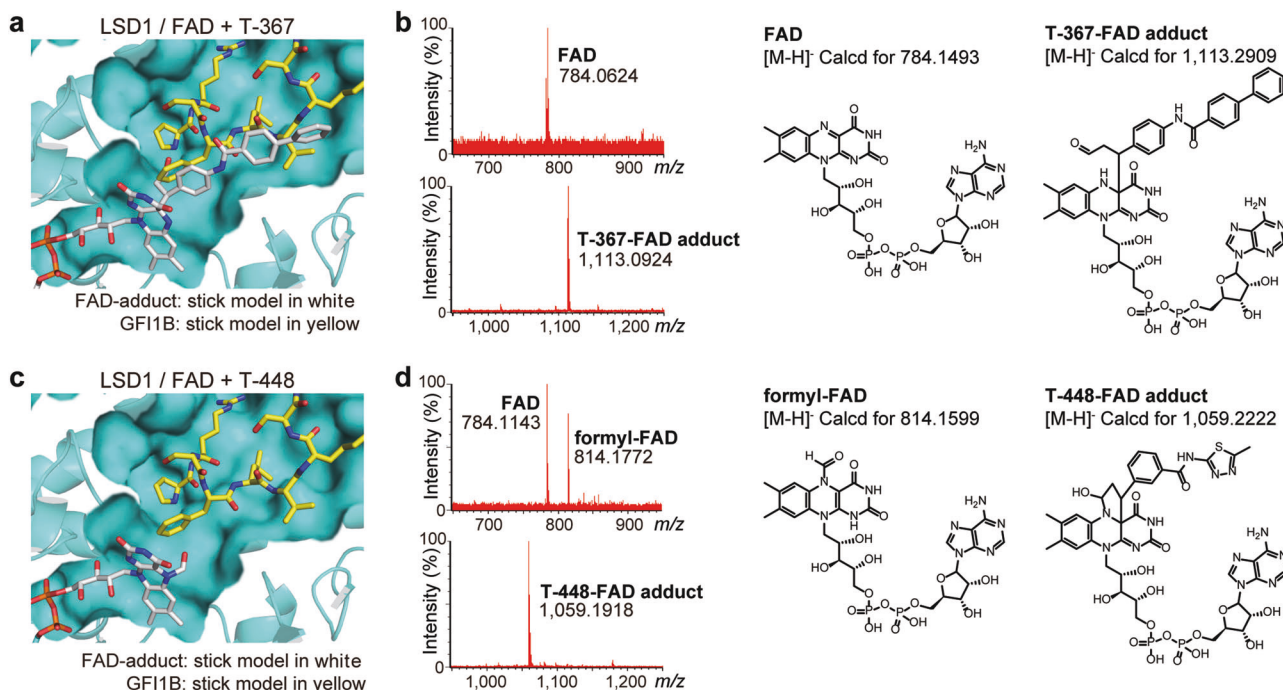
adduct is an intermediate product toward formyl-FAD generation (Fig. S7). The resultant formyl-FAD adduct was compact (Fig. 3c, stick model in white), and seemed to show no steric clash with the GFI1B peptide in the active site of LSD1 (Fig. 3c, stick model in yellow). The LC/MS peak with  $m/z$  814 was not detected upon T-367 treatment (Fig. 3d). These data support the hypothesis that T-448 inactivates LSD1 enzyme activity through generating a compact formyl-FAD adduct, and the formation of formyl-FAD adduct has minimal impact on the interaction between LSD1 and GFI1B.

T-448 increases H3K4 methylation in the brain without causing hematological side effects

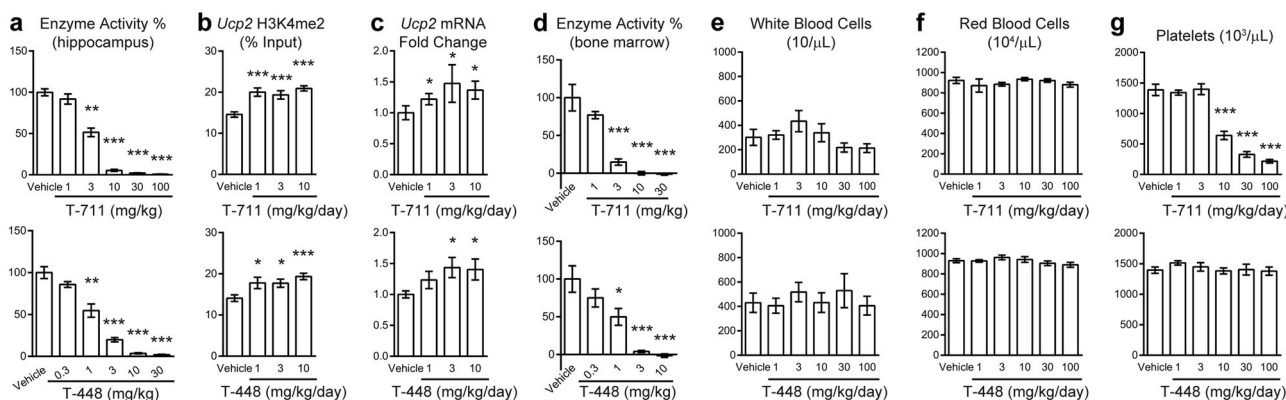
In vivo inhibition of LSD1 enzyme activities by T-711 or T-448 can be accurately determined by an ex vivo LSD1 enzyme assay due to their irreversible nature in LSD1 inhibition. Both T-711 and T-448 showed a similar half-life in PK studies in ICR mice;  $T_{max}$  values of both compounds was achieved ~1 h after oral administration in ICR mice, and then plasma concentrations declined in a time-dependent manner (Fig. S8). We collected tissues 2 h after the administration of these compounds and conducted ex vivo LSD1 enzyme assays.

Cortex and hippocampus were selected as representative brain regions that are critical for learning and memory functions. The inhibition of LSD1 enzyme activities by T-711 and T-448 in the brain were examined in ICR mouse hippocampus. Both compounds showed dose-dependent inhibition of LSD1 enzyme activities with full inhibition at 10 mg/kg in the mouse hippocampus (Fig. 4a). The effects of these compounds on H3K4me2 levels at the upstream region of the *Ucp2* gene in the mouse cortex were determined after a 9-day repeated administration at up to 10 mg/kg. Both compounds significantly increased the H3K4me2 levels at all doses tested (Fig. 4b). The effects of these compounds on gene expression were examined in the same experimental paradigm; the significant induction of *Ucp2* mRNA was observed after administration of T-711 (significant at 1 mg/kg/day or higher) and T-448 (significant at 3 mg/kg/day or higher) in the mouse hippocampus (Fig. 4c).





**Fig. 3** T-448 produced a compact formyl-FAD adduct in LSD1. **a** Superimposed structures of T-367-FAD adduct in human LSD1 by crystal analysis (stick model in white) and of N-terminal GF11B peptide (PRSFVL) in the LSD1-complex by modeling (stick model in yellow). **b** LC/MS spectra of FAD and FAD-adducts extracted from human recombinant LSD1 treated with T-367. **c** Superimposed structures of formylated FAD in LSD1 generated after T-448 treatment by crystal analysis (stick model in white) and N-terminal GF11B peptide (PRSFVL) in the LSD1-complex by modeling (stick model in yellow). **d** LC/MS spectra of FAD and FAD-adducts extracted from human recombinant LSD1 treated with T-448



**Fig. 4** T-448 increased brain H3K4me2 levels without hematological safety issue in mice. **a** LSD1 enzyme activities in the mouse hippocampus at 2 h after the single administration of compounds.  $n = 6$ . One-tailed Shirley-Williams test vs vehicle-treated groups,  $**p < 0.005$ ,  $***p < 0.0005$ . **b**, **c** H3K4me2 level at the upstream region of the *Ucp2* gene in the mouse cortex (**b**) and the expression level of *Ucp2* mRNA in the mouse hippocampus (**c**) 1 day after the 9-day repeated administration.  $n = 9$ . One-tailed parametric Williams' (b) or Shirley-Williams test (c) vs vehicle-treated groups,  $*p < 0.025$ ,  $***p < 0.0005$ . **d** LSD1 enzyme activities in the mouse bone marrow at 2 h after the single administration.  $n = 7$  (vehicle-treated groups) or 4 (compound-treated groups). One-tailed parametric Williams' test vs vehicle-treated groups,  $*p < 0.025$ ,  $***p < 0.0005$ . **e-g** Numbers of white blood cells (**e**), red blood cells (**f**), and platelets (**g**) in the whole blood 1 day after the 4-day repeated administration.  $n = 6$ . Two-tailed Shirley-Williams (f, T-711) or parametric Williams' test (other Figures) vs vehicle-treated groups,  $***p < 0.0005$ . All graphs indicate mean  $\pm$  SEM

We characterized *in vivo* hematological side effects of these compounds in ICR mice. The inhibition of LSD1 enzyme activity in hematopoietic lineage cells was determined by *ex vivo* enzyme assays using mouse bone marrow tissues. As shown in Fig. 4d, dose-dependent LSD1 inhibition by both T-711 and T-448 in the bone marrow was observed; T-711 at 10 mg/kg and T-448 at 3 mg/kg fully inhibited LSD1 in the bone marrow. The effects of these compounds on blood cell counts (number of white blood cells, red blood cells, and platelets) in mouse peripheral blood were evaluated after a 4-day repeated administration. T-711 at 10 mg/

kg/day or higher caused a significant decrease in platelet numbers, while T-448 at up to 100 mg/kg/day did not cause any change in blood cell numbers (Fig. 4e–g). Two-week repeated treatment with T-711 at 30 mg/kg caused a significant and remarkable reduction in both red blood cells and platelets, whereas two-week repeated treatment with T-448 at 100 mg/kg caused a significant, but limited (14%) reduction in red blood cells without affecting white blood cells and platelets (Fig. S9). These results suggest the superior hematological safety profile of T-448 in ICR mice.

T-448 restores learning function through modulating H3K4 methylation levels

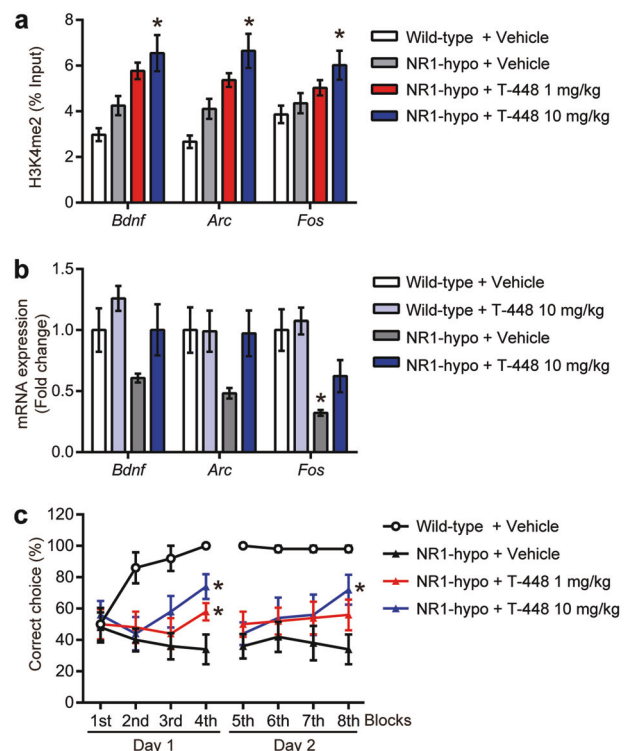
The NMDAR plays a pivotal role in memory formation through promoting the expression of synaptic plasticity-related genes such as *Bdnf*, *Arc*, and *Fos*. Regulation of *Bdnf*, *Arc*, and *Fos* mRNA expression is also controlled by epigenetic mechanisms [1, 34–37]. Therefore, we used *Nr1<sup>neo-/-</sup>* (NMDA receptor NR1 subunit hypofunction: NR1-hypo) mice to assess the therapeutic potential of T-448. Administration of T-448 to NR1-hypo mice at 1 mg/kg/day (49% inhibition in ICR mouse brain) and 10 mg/kg/day (96% inhibition in ICR mouse brain) for 3 weeks dose-dependently increased the H3K4me2 levels around *Bdnf*, *Arc*, and *Fos* genes in the mouse hippocampus. The difference was significant at 10 mg/kg/day (Fig. 5a). In contrast to H3K4me2 methylation, basal levels of *Bdnf*, *Arc*, and *Fos* gene mRNA were decreased in NR1-hypo mouse brains compared to wild-type mice (Fig. 5b). Treatment with T-448 did not change in *Bdnf*, *Arc*, and *Fos* mRNA levels in wild-type mouse brains, but exhibited a tendency to restore normal levels in NR1-hypo mouse brains at 10 mg/kg/day (Fig. 5b).

NR1-hypo mice exhibited learning deficits in correct choices in the water Y-maze test (Fig. 5c). Repeated administration of T-448 for 3 weeks resulted in partial but statistically significant and dose-dependent rescue effects on the rate of correct choices in NR1-hypo mice (Fig. 5c). Taken together, these observations suggest that T-448 partially restored learning impaired by NMDAR hypofunction by increasing H3K4 methylation in the brain and consequently normalizing levels of neural plasticity-related gene expression.

DISCUSSION

Aberrant changes in gene expression can be associated with neurodevelopmental and neuropsychiatric disorders. In addition to genetic mutations, “gene-environment” interactions and epigenetic control is critical in brain function [38]. Organisms can adjust gene expression in response to intrinsic and environmental signals through epigenetic mechanisms, such as modification of chromatin structure and DNA methylation. Thus, dysfunction of epigenetic mechanisms may also underlie pathophysiology of CNS disorders [39, 40]. Among epigenetic pathways, H3K4 methylation is critically involved in various neural functions, such as learning and memory. For example, haploinsufficiency of the *CHD8* (chromodomain helicase DNA-binding protein 8) gene, which encodes for a methylated histone reader, has been implicated as a cause of ASD [41, 42], suggesting that upregulation of H3K4 methylation may be a promising strategy to treat CNS disorders.

In this study, we discovered a novel LSD1 inhibitor, T-448, which increased H3K4me2 levels in the brain and improved learning in an NMDAR hypofunction mouse model without causing any change in blood cell numbers at the effective dose (10 mg/kg), suggesting that it has a wider safety margin against thrombocytopenia than do other known LSD1 inhibitors. This improved safety profile of T-448 would be due to its minimal impact on the LSD1-GFI1B complex and the lack of *Gfi1* mRNA induction in TF-1a cells. Co-crystal structural and LC/MS analyses revealed that T-367, the eutomer of T-711, produced a bulky FAD-adduct causing a steric clash with the GFI1B peptide. This steric interference may lead to the dissociation of the LSD1-GFI1B complex and *Gfi1* mRNA induction in TF-1a cells. In contrast, T-448 produced a compact formyl-FAD adduct that could avoid the steric clash with cofactor GFI1B. As for the molecular mechanism, the production of formyl-FAD adduct would be mediated by the transient generation of full T-448-FAD adduct, because LC/MS analysis detected the time-dependent decrease of full T-448-FAD adduct (*m/z* 1,059) and the time-dependent increase of formyl-FAD (*m/z* 814). In line with this hypothesis, SPR analysis showed the transient inhibition of the LSD1-GFI1B interaction by T-448.



**Fig. 5** T-448 treatment for 3 weeks increased brain H3K4me2 levels and restored learning function in NMDAR hypofunction mice. **a** H3K4me2 levels around *Bdnf*, *Arc*, and *Fos* genes in the wild-type and the NR1-hypo mouse hippocampus. Graphs indicate mean ± SEM. *n* = 5. One-tailed parametric Williams’ test vs vehicle-treated NR1-hypo mouse group, \**p* < 0.025. **b** mRNA expression levels of *Bdnf*, *Arc*, and *Fos* genes in the wild type and the NR1-hypo mouse hippocampus. Graphs indicate mean ± SEM. *n* = 4. Welch’s test vs vehicle-treated wild-type mouse group, \**p* < 0.05. **c** Effects of T-448 on the learning behavior of NR1-hypo mice in water Y-maze test. Correct choice percentage was calculated in every block. Data are shown as mean + SEM. *n* = 10. One-tailed parametric Williams’ test vs vehicle-administered group at 4th and 8th blocks, \**p* < 0.025

Further studies are needed to understand these mechanisms of action in detail.

Platelet reduction after treatment with an LSD1 inhibitor that disrupts LSD1-complex was observed for at least 3 days after administration in mice [23]. If the transient disruption of the LSD1-GFI1B interaction by T-448 caused platelet reduction, the blood cell examination performed 1 day after repeated administration for 4 days would reveal the changes in platelet numbers. Under the experimental conditions, T-711, but not T-448, reduced platelet numbers. This suggests that the transient disruption of the LSD1-GFI1B interaction by T-448 may not be associated with hematological adverse effects.

The discovery of T-448 enabled the in vivo characterization of therapeutic effects by the specific inhibition of LSD1 enzyme activity. NMDAR-dependent signal plays pivotal roles in the transcription of neural plasticity-related genes, such as *Bdnf*, *Arc*, and *Fos* [1]. In fact, reduced transcription level of *Fos* gene was observed in NR1-hypo mouse brain [28, 29]. The increasing trend in H3K4me2 levels in NR1-hypo mice may be a compensatory mechanism for the impaired transcription. Repeated administration of T-448 at 10 mg/kg enhanced the H3K4me2 levels around *Bdnf*, *Arc*, and *Fos* genes. These findings suggested that LSD1 inhibition restores cellular responsiveness to NMDAR-dependent signals via increasing the H3K4 methylation. Furthermore, T-448 at 10 mg/kg rescued the learning deficits in NR1-hypo mice. Inhibition of LSD1 enzyme activity could be an approach to treat

diseases associated with NMDAR hypofunction such as schizophrenia and ASD.

As discussed, epigenetic mechanisms may have pivotal roles in responding to environmental changes through the modification of gene expression. In other words, modulation of cell-intrinsic signals or extracellular signals alone by the stimulation of signal transduction molecules or receptors would not be enough to fully modify disease pathophysiology with epigenetic dysfunction. Therefore, environmental factors should be carefully considered when assessing the therapeutic potential of T-448. In addition to H3K4 demethylation, LSD1 is also involved in the demethylation of H3K9 [11] and non-histone proteins, such as DNMT1 [12] and p53 [43]. These molecules are also involved in neurocognitive function and associated with neurodevelopmental disorders [44, 45]. Thus, T-448 may produce its pharmacological effects through the modulation of both histone and non-histone substrates.

In summary, we discovered a novel LSD1 enzyme inhibitor, T-448, which has a minimal impact on the LSD1-GFI1B complex and on hematological liability in mice through the generation of a compact formyl-FAD adduct. T-448 increased brain H3K4 methylation levels and restored learning function in mice with NMDAR hypofunction. T-448-type LSD1 inhibitors with improved safety profiles can provide unique therapeutic opportunities for CNS disorders with epigenetic dysregulation.

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## AUTHOR CONTRIBUTIONS

S.Matsuda and H.Kimura conceived and designed the experiments. S.Matsuda, R.B., H.O., S.Igaki, Y.K., S.Iwasaki, K.M., R.Hibino, H.Kamada, T.H., M.Iwatani, K.T., and R.Hara performed the experiments and analyzed the data. S.Morimoto, M.T., and M.Ito designed and synthesized T-448. S.Matsuda and H.Kimura wrote the manuscript.

## ADDITIONAL INFORMATION

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