Examination of the hippocampal contribution to
serotonin 5-HT_{2A} receptor-mediated facilitation of object memory in
C57BL/6J mice

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ABSTRACT

The rodent hippocampus supports non-spatial object memory. Serotonin 5-HT_{2A} receptors (5-HT_{2A}R) are widely expressed throughout the hippocampus. We previously demonstrated that the activation of 5-HT_{2A}Rs enhanced the strength of object memory assessed 24 h after a limited (i.e., weak memory) training procedure. Here, we examined the subcellular distribution of 5-HT_{2A}Rs in the hippocampal CA1 region and underlying mechanisms of 5-HT_{2A}R-mediated object memory consolidation. Analyses with immuno-electron microscopy revealed the presence of 5-HT_{2A}Rs on the dendritic spines and shafts, and presynaptic terminals of hippocampal CA1 neurons. In an object recognition memory procedure that places higher demand on the hippocampus, only post-training systemic or intrahippocampal administration of the 5-HT_{2A}R agonist TCB-2 enhanced object memory. Object memory enhancement by TCB-2 was blocked by the 5-HT_{2A}R antagonist, MDL 11,937. The memory-enhancing dose of systemic TCB-2 increased extracellular glutamate levels in hippocampal dialysate samples, and increased the mean in vivo firing rate of hippocampal CA1 neurons. In summary, these data indicate a pre- and post-synaptic distribution of 5-HT_{2A}Rs, and activation of 5-HT_{2A}Rs selectively enhanced the consolidation of object memory, without affecting encoding or retrieval. The 5-HT_{2A}R-mediated facilitation of hippocampal memory may be associated with an increase in hippocampal neuronal firing and glutamate efflux during a post-training time window in which recently encoded memories undergo consolidation.

1. Introduction

The rodent hippocampus supports spatial memory as well as non-spatial object or item memory (Cohen et al., 2013). We previously reported that silencing hippocampal neuronal activity via local administration of the GABA-A receptor agonist muscimol, impaired the encoding, consolidation and retrieval of novel object memory in mice (Cohen et al., 2013). The hippocampus receives serotonergic projections from midbrain raphe nuclei and serotonergic neurotransmission modulates hippocampal plasticity, electrical activity, hippocampal-dependent behaviors and cognitive performance (Nichols and Nichols, 2008). Many antidepressants and hallucinogens function by targeting serotonergic system, especially 5-HT receptors (Schmitt et al., 2006; Seeman, 2002). Recently, the serotonin 5-HT_{2A} receptor (5-HT_{2A}R) has received considerable attention for its contribution to cognition, decision-making and execution, hallucination and psychiatric disorders (Zhang and Stackman, 2015). It is of interest to define the specific neural circuits and neurophysiological mechanisms by which 5-HT_{2A}Rs influence distinct behaviors. As a member of the G protein-coupled receptor family, 5-HT_{2A}Rs are distributed extensively in the brain, especially in the hippocampus and frontal cortex (Nichols and Nichols, 2008). The current study addressed whether the serotonergic input to 5-HT_{2A}R-expressing hippocampal neurons affects the distinct object memory processes of encoding, consolidation and retrieval. We also examined the influence of 5-HT_{2A}Rs on physiological mechanisms that contribute to hippocampal memory.

Administration of 5-HT_{2A}R agonists has been shown to enhance memory in rats (Alhaider et al., 1993), monkeys (Williams et al., 2002) and humans (Nic Dhonnchadha and Cunningham, 2008). We have previously found that mice administered the high affinity 5-HT_{2A}R agonist TCB-2 after a minimal object training session exhibited enhanced memory during a retention test

24 h later, as compared to control-treated mice (Zhang et al., 2013). The object recognition task relies on the rodent's inherent preference for exploring novel stimuli, and permits operationally defining object memory encoding as that occurring during a sample session (i.e., training), object memory consolidation as occurring after the sample session, and object memory retrieval occurring during the test session (Clark et al., 2000). Object recognition memory processes depend on the intact function of the dorsal hippocampus (Cohen et al., 2013; Hammond et al., 2004). Immunohistochemical evidence indicates a rich distribution of 5-HT_{2A}R in the hippocampus (Luttgen et al., 2004), but the subcellular distribution of 5-HT_{2A}R in the hippocampus is not fully understood. Although we have reported that systemic activation of 5-HT_{2A}R enhanced object memory in mice (Zhang et al., 2013), the underlying physiological mechanisms by which 5-HT_{2A}Rs might influence hippocampal-dependent object memory, and the influence of 5-HT_{2A}R on the encoding and retrieval of object memory remain elusive. To determine the subcellular distribution of 5-HT_{2A}Rs and mechanisms of 5-HT_{2A}R on hippocampal-dependent object memory, we employed electron microscopy, behavioral, neurochemical, and electrophysiological approaches. Our results indicate a pre- and post-synaptic distribution of 5-HT_{2A}Rs, and that only the post-training activation of 5-HT_{2A}R affects hippocampal-dependent object memory. These results are consistent with the view that 5-HT_{2A}Rs modulate post-training hippocampal physiological processes that support memory consolidation.

2. Materials and Methods

2.1. Animals

Adult male C57BL/6J mice (8-12 weeks) from Jackson Laboratory (Bar Harbor, ME) were housed in groups of 4 per standard polycarbonate cage with *ad libitum* access to food and water. Mouse cages were maintained in a temperature- and humidity-controlled vivarium with a 12-h light/12-h dark cycle (lights on at 7:00 AM). All behavioral testing took place during the light phase, from 12:00 PM to 5:00 PM. All procedures were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the Institutional Animal Care and Use Committee approval.

2.2. Drugs

TCB-2 and MDL 11,939 were purchased from Tocris (Ellisville, MO). TCB-2 was dissolved in sterile water first and then diluted with the same volume of 1.8% NaCl solution. A 1% solution of DMSO in sterile saline served as the vehicle for MDL 11,939 (0.5 mg/kg, i.p.). The volume for systemic injection was 1.0 ml per 100 g of body weight.

2.3. Immunohistochemistry for electron microscopy

Mice received an injection of ketamine–xylazine anesthesia cocktail (1:1; 0.1 ml/kg, i.p.) and then were transcardially perfused with ice-cold fixative containing 4% paraformaldehyde, with 0.05% glutaraldehyde and 15% (v/v) saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, brains were removed and immersed in the same fixative for 2 h. Tissue blocks containing the dorsal hippocampus were washed thoroughly in 0.1 M PB. Coronal 60-nm thick sections were cut on a Vibratome (Leica V1000).

Immunohistochemical reactions at the electron microscopic (EM) level were carried out using a pre-embedding immunogold method described previously (Luján et al., 1996). Briefly, free-floating sections were incubated in 10% normal goat serum (NGS) diluted in Tris-buffered saline. Sections were then incubated in anti-5-HT_{2A}R antibody (PC176, EMD Millipore, Billerica, Massachusetts) at 3-5 µg/ml diluted in 1% NGS/Tris-buffered saline, followed by incubation in goat anti-rabbit IgG coupled to 1.4 nm gold (Nanoprobes, Stony Brook, NY), diluted 1:100 in 1% NGS/Tris-buffered saline. Sections were postfixated in 1% glutaraldehyde and washed in double-distilled water, followed by silver enhancement of the gold particles with a HQ Silver kit (Nanoprobes). Sections were then treated with osmium tetroxide (1% in 0.1 M PB), block-stained with uranyl acetate, dehydrated in graded series of ethanol, and flat-embedded on glass slides in Durcupan (Fluka) resin. Regions of interest were cut at 70-90 nm on an ultramicrotome (Reichert Ultracut E, Leica, Austria) and collected on 200-mesh copper grids. Staining was performed on drops of 1% aqueous uranyl acetate followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a Jeol-1010 electron microscope.

2.4. Spontaneous object recognition memory task and systemic drug administration.

Our goal was to determine the selective influence of systemic TCB-2 on distinct memory processes of encoding, consolidation, and retrieval. Therefore, we employed a version of the spontaneous object recognition memory procedure that yields a strong, long-term object memory (Vick et al., 2010), which places higher demands on hippocampal function (Cohen et al., 2013; Hammond et al., 2004). In brief, during the sample session, two identical toy objects (zinc-plated cabinet leveling feet, 4.2 cm dia and 6.0 cm tall) were placed in opposite corners (NW and SE) of the arena. Each mouse was allowed a maximum of 10 min to accumulate 38 s of exploration of either sample object, or a total of 30 s of exploration of each sample object. This 30/38-s

sample session training procedure promotes the encoding and consolidation of a strong, long-term object memory, which then guides significant preferential exploration of the novel object during a test session 24 h later (Cohen and Stackman, 2015; Stackman et al., 2002; Vick et al., 2010). In our previous study testing the memory-enhancing properties of TCB-2 (Zhang et al., 2013), mice were only permitted 15 s of exploration of either sample object – a procedure typically resulting in weak object memory in control mice 24 h later. For the present study, mice were returned to the same arena 24 h after the sample session for the test session, in which one of the familiar objects was replaced with a novel object (plastic toy gorilla, approximately 5.0 cm long and 6.0 cm tall). The novel toy object was placed in the same location as the object it replaced (NW or SE) to reduce spatial significance of the objects. Mice were permitted to freely explore the novel and familiar objects during the 5-min test session. The discrimination ratio, a measure of object memory, was calculated for each mouse by subtracting the time spent exploring the familiar object from the time spent exploring the novel object and dividing the result by the total time spent exploring both objects. Discrimination ratio scores range from -1 to 1, with 0 indicating equal or chance exploration levels. A positive discrimination ratio indicates preference for exploring the novel object, while a negative discrimination ratio indicates preference for exploring the familiar object. For our systemic TCB-2 study, each mouse was randomly assigned to one of four treatment groups - receiving injections of vehicle or TCB-2 20 min before the sample session, right after the sample session, and 20 min before the test session presented 24 h after the sample session. This *before sample/after sample/before test* design yielded the following treatment groups: vehicle/vehicle/vehicle (a common control group), TCB-2/vehicle/vehicle (to assess memory encoding), vehicle/TCB-2/vehicle (to assess memory consolidation), and vehicle/vehicle/TCB-2 (to assess memory retrieval).

2.5. Intra-hippocampal microinfusion

Mice were implanted with chronic bilateral guide cannulae (Plastics One, Roanoke, VA) above the CA1 region of dorsal hippocampus (A/P - 2.0 mm, M/L $\pm$ 1.5 mm, D/V - 1.1 mm from bregma) (Franklin and Paxinos, 2008). Mock infusions were given each day for the 2 days prior to the actual intra-hippocampal microinfusion, to habituate the mice to the restraint and manipulation necessary to perform the bilateral microinfusion. Mice received bilateral (0.5 μ l/side, 0.167 μ l/min) intra-hippocampal TCB-2 (2 μ g/ μ l in artificial cerebrospinal fluid (aCSF)) right after the sample session. The test session was presented 24 h after the microinfusion was completed.

2.6. Microdialysis and extracellular glutamate assay

Under deep isoflurane anesthesia, mice were implanted with a unilateral guide cannula over the right CA1 region of dorsal hippocampus, and then recovered for 7 days. Eighteen hours after a sample session, the microdialysis probe (cut-off 18 kDa, 0.5 mm membrane) was inserted through the guide cannula and secured in place. The probe inlet was perfused with artificial CSF (aCSF) at a flow rate of 1.2 μ l/min. After 6 h of equilibration, three 10- μ l samples were collected at an interval of 10 min to obtain basal extracellular glutamate level from dorsal hippocampus and five samples were collected after systemic administration of drugs.

Dialysate samples were analyzed by HPLC with electrochemical detection. Samples were first added to 10 μ l of derivatization reagent (2-mercaptoethanol in 1 ml O-phthaldialdehyde solution, pH 9.5). Ten μ l of derivatized solution was manually injected into a reverse-phase microsphere column (2 $\times$ 100 mm, packed with a C18, 3 μ m particle size, Agilent). A working electrode (MF-1000, BAS) was set at +700 mV vs. an Ag/AgCl reference electrode. Mobile phase (0.1 M phosphate buffer, pH 6.0 with 5% methanol and 40 mg/L EDTA) was delivered by a LC-10AT

Shimadzu pump at 400 μ l/min. Glutamate was estimated by comparing peak areas between microdialysis samples and a known standard using Powerchrom V2.2 software. The mean relative recovery efficiency of the microdialysis probe was 8% during *in vitro* assessment.

2.7. Single-unit recording

Under deep isoflurane anesthesia, mice were implanted with custom-made microdrives containing four tetrodes (each a twisted bundle of four 25- μ m nichrome wires, California Fine Wire). After 1-wk recovery, tetrodes were advanced ventrally by 50-75 μ m/day. Signals from tetrodes were unity-gain amplified at the mouse's head and passed through a commutator to a preamplifier unit. Signals were bandpass filtered at 150-8000 Hz; digitized at 40 kHz were recorded with a MAP system (Plexon, Dallas, TX) and analyzed by manual and automatic sorting with Offline Sorter software (v3.2.1, Plexon). An overhead camera acquired the x,y coordinates of two LEDs fixed to the headstage at the end of the recording cable. The position coordinates were acquired by CinePlex digital videotracking software (Plexon) and then stored for analysis. Neuronal activity was monitored during 10-20 min cell screening sessions while mice moved freely about the floor of the high-walled cylinder (44 cm dia. by 36 cm tall) constructed of white polyethylene. Recording sessions began when sufficient numbers of hippocampal CA1 pyramidal cells were detected as exhibiting characteristic complex-spike bursts and spiking modulated by the theta rhythm. If the firing properties of the units proved to be stable, a series of 15-min recording sessions were initiated to test the influence of systemic TCB-2 on the firing rates of a common set of CA1 neurons from freely moving mice. Three mice received a systemic injection of saline (0.9%, 1 ml/ 100 g, IP) before the first recording session, and then received a systemic injection of TCB-2 (1 mg/kg, 1 ml/100 g, IP). Two mice received the same injections in reverse order.

2.8. *Waveform clustering*

Spikes were sorted through a process of waveform clustering within the Offline Sorter program (Plexon). The same criteria used in identifying spike clusters from the first injection recording session were applied to the recording session after the second injection. The data for a given cell were only included in the analysis if we could identify the same cell on the same tetrode, and with the same cluster boundaries in both recording sessions. In cases in which the tetrodes were not stable, for example, in situations in which the tetrodes may have changed position in the brain as evidenced by the lack of proper waveform matching between the two recording sessions, the data for that mouse were dropped from the analysis. Only units with clear boundaries and less than 0.5% of spike intervals within a 1-ms refractory period were included in the following analyses. The putative pyramidal neurons were included in analyses if they had: 1) low baseline firing rate (0.5-10 Hz) and irregular firing pattern; 2) dominant short interspike interval (3-10 ms); and 3) a wide waveform ($> 300 \mu\text{sec}$). The putative interneurons were classified, accordingly, as having relatively narrow waveforms ($< 250 \mu\text{sec}$) and high firing rates ($> 5 \text{ Hz}$), and the interspike interval histograms exhibited a later peak and a much slower decay compared to putative pyramidal neurons. Spike duration is defined by the latency of spike peak to trough. Sorted neural spikes were analyzed with NeuroExplorer (V3.266, Nex Technologies, Littleton, MA). Neurons were included in the analyses of TCB-2 effects on firing rate only if the spike sorting routines revealed consistent clusters cut for each respective recording session. The spike data for any neuron that failed to be represented in the second recording session, were dropped from the analysis.

2.9. *Histology*

Cannulae, microdialysis probe and tetrode placements were confirmed by examination of Cresyl violet stained 50- μ m coronal sections with light microscopic methods. The data for any mice determined to have inappropriate placement were excluded from the analyses.

2.10. Data analysis

Data were expressed as mean $\pm$ SEM. Two-way repeated measures ANOVA of raw data with time as a repeating factor and drug dose as dependent factor were used to evaluate glutamate level measurement. One-way ANOVA and independent samples Student *t*-tests were used to analyze the object recognition behavioral data and neuronal firing rates. The significance level was set at $p < 0.05$. All statistical tests were conducted using the SPSS v19 (IBM, Armonk, NY) software package.

3. Results

3.1. Immunoelectron microscopy reveals a presynaptic and postsynaptic distribution of 5-HT_{2A}Rs in the CA1 pyramidal cell layer of the dorsal hippocampus

The precise subcellular distribution of 5-HT_{2A}Rs within the hippocampal cell fields has not previously been defined. High-resolution immunoelectron microscopy was used to determine the subcellular localization of 5-HT_{2A}R in the CA1 pyramidal cell layer of the dorsal hippocampus. The pre-embedding immunogold method revealed that immunoparticles for the 5-HT_{2A}R were found primarily along the plasma membrane of dendritic shafts and dendritic spines and at intracellular sites within the postsynaptic dendrites (Figs. 1A-E). Immunoparticles for the 5-HT_{2A}Rs were also found at pre-synaptic sites – on axon terminals of excitatory synapses with dendritic spines of CA1 pyramidal cells (Fig. 1F). The pattern of subcellular distribution of 5-HT_{2A}Rs reveals that 5-HT_{2A}Rs are positioned to affect hippocampal-dependent memory processes via modulation of both postsynaptic and presynaptic mechanisms.

3.2. Activation of 5-HT_{2A}R with TCB-2 enhances the consolidation of object memory

We tested the influence of systemic activation of 5-HT_{2A}R with TCB-2 on object memory processes using a procedure modified from that used previously (Zhang et al., 2013). Briefly, mice explored two novel objects during a sample session, and during a test session 24-h later in which one of the sample objects was replaced with a novel object. To assess the selective effects of TCB-2 (1.0 mg/kg, i.p.) on encoding, consolidation, and retrieval of object memory, mice received drug or vehicle before the sample session, after the sample session, or right before the test session, respectively. The measures of latency to achieve the sample object exploration criteria during the sample session were not significantly affected by treatment, $F(3, 38) = 0.91$, $P > 0.05$. Respective group mean $\pm$ S.E.M. were as follows: control, 384.83 ± 27.83 s ($n = 10$);

TCB-2 before sample session, 398.59 ± 23.55 s ($n = 10$); TCB-2 after sample session, 346.38 ± 23.53 s ($n = 12$); and TCB-2 before test session, 360.86 ± 24.58 s ($n = 10$). Moreover, there was no difference in distance traveled and velocity between each group during the arena habituation, sample session or test session (see Supplementary Materials Fig. S1). These results indicate that TCB-2 does not affect the ambulatory behavior and motivation of mice to explore the sample objects under our experimental protocol. A one-way ANOVA on test session discrimination ratio scores yielded a significant main effect of treatment group, $F(3, 38) = 3.90$, $P < 0.05$. *Post-hoc* LSD multiple comparisons indicated that mice that received TCB-2 after sample session exhibited a significantly higher discrimination ratio score than that of the control ($P < 0.05$; Fig. 2A). There were no significant differences between the control groups and the TCB-2 before sample session or the TCB-2 before test session (all P values > 0.05). To further examine the effect of TCB-2 on object memory consolidation, a second cohort of mice received 5 mg/kg TCB-2 right after the sample session. As described in Fig. 2B, TCB-2 induced a dose-dependent increase in discrimination ratio ($F(2, 28) = 6.41$, $P < 0.01$). *Post-hoc* LSD tests indicated that 5 mg/kg TCB-2 ($P < 0.01$; $n=9$) induced a higher discrimination ratio than did 1 mg/kg TCB-2 ($P < 0.05$) compared to saline mice. These results indicate that TCB-2-enhanced the consolidation of object memory and demonstrate that the encoding and retrieval of object memory are not influenced by systemic activation of 5-HT_{2A}Rs. We previously reported that administration of TCB-2 enhanced the strength of object memory in mice trained in a procedure that leads to weak object memory performance in control-treated mice (Zhang et al., 2013). The present results extend our earlier report by providing further evidence that consolidation of object memory is selectively facilitated by systemic TCB-2.

Next, the 5-HT_{2A}R specificity of the TCB-2-induced enhancement of memory consolidation was tested in a naïve cohort of mice using the 5-HT_{2A}R antagonist MDL 11,939 (0.5 mg/kg, i.p.). For each group the first injection was administered immediately after the sample session, and second injection was given 10 min later. Latency to achieve the sample object exploration criteria were not significantly affected by treatment, $F(2,25) = 7.45$, $P > 0.05$; with mean $\pm$ S.E.M. as follows: vehicle + saline, 391.24 ± 17.63 s ($n = 10$); vehicle + TCB-2, 315.78 ± 35.58 s ($n = 9$); and MDL 11,939 + TCB-2, 427.67 ± 42.19 ($n = 9$). As above, vehicle + TCB-2 mice exhibited a greater preference for exploring the novel object during the test session, and MDL 11,939 pretreatment completely blocked the TCB-2-induced memory enhancement. A one-way ANOVA on discrimination ratio measures yielded a significant effect of treatment group ($F(2,25) = 7.45$, $P < 0.05$; Fig. 2C). *Post-hoc* LSD multiple comparisons indicated that TCB-2 enhanced the discrimination ratio as compared to the vehicle + saline group ($P < 0.01$), while MDL 11,939 pretreatment prevented the enhancement of object memory induced by TCB-2 (MDL 11,939 + TCB-2 vs. vehicle + TCB-2, $P < 0.01$). There were no differences between the vehicle + saline group and the MDL 11,939 + TCB-2 group ($P < 0.05$). Altogether, the results further confirm that selective activation of 5-HT_{2A}R after the sample session enhances the consolidation of object memory.

To determine whether hippocampal 5-HT_{2A}Rs contribute to the memory enhancing effects of TCB-2, a cohort of naïve mice ($n = 20$) received bilateral microinfusion of TCB-2 (1 μ g/0.5 μ l per side) or aCSF (0.5 μ l per side) into the CA1 region of dorsal hippocampus right after the sample session, and novel object memory was tested 24 h later. The data for 3 mice were excluded from analyses due to misplacement of cannulae/injection sites. Mice that received bilateral intra-CA1 microinfusion of TCB-2 spent significantly more time exploring the novel

object during the test session than did the mice that received intra-CA1 aCSF. An independent groups Student's *t*-test yielded a significant difference in discrimination ratio scores between the two groups of mice ($t(15) = -3.36$, $P < 0.001$; Fig. 2D). These data indicate that selective post-sample activation of hippocampal 5-HT_{2A}R enhances novel object memory consolidation. For a representative example of histological confirmation of cannula placement, and the comprehensive map of injection sites, see Fig. 2E and 2F, respectively.

3.3. Systemic activation of 5-HT_{2A}R with TCB-2 increases extracellular glutamate efflux from dorsal hippocampus

We tested whether manipulation of 5-HT_{2A}R altered extracellular glutamate levels in microdialysis samples from the dorsal hippocampus of male C57BL/6J mice. The pre-drug baseline glutamate levels observed in dialysate samples did not differ between treatment groups ($t(13) = -0.22$, $P > 0.05$; mean $\pm$ S.E.M. for vehicle 0.15 ± 0.027 μ M ($n = 8$) and TCB-2 0.15 ± 0.03 μ M ($n = 9$). TCB-2 (1 mg/kg, i.p.) significantly increased the glutamate levels in dialysate samples. A two-way repeated measures ANOVA on glutamate levels indicated a significant main effect of treatment ($F(1, 13) = 23.72$, $P < 0.001$), a significant main effect of time ($F(6, 78) = 2.44$, $P < 0.05$) and a significant main effect of treatment $\times$ time interaction ($F(6, 78) = 2.89$, $P < 0.05$; Fig. 3A). The level of extracellular glutamate in hippocampal dialysate samples was significantly elevated from 20 to 40 min after systemic TCB-2 administration.

3.4. Systemic activation of 5-HT_{2A}R with TCB-2 increases the mean firing rates of pyramidal neurons in CA1 region of dorsal hippocampus

CA1 neurons were recorded from individual mice during two 15 min sessions of free movement about a cylindrical arena. Each recording session was preceded by a systemic injection of saline vehicle or TCB-2 (1.0 mg/kg, i.p.). Three mice received the saline treatment first, and then TCB-

2, while two mice were treated with TCB-2 and then saline. The mean firing rate of the CA1 neurons after saline treatment was 0.89 ± 0.10 Hz, and 1.33 ± 0.16 Hz after TCB-2 treatment. There was no effect of order of injection on the drug influence on neuronal firing rate. A Student's *t*-test revealed a significant difference in CA1 neuronal firing rates under the two treatment conditions ($t(32) = -2.32$, $P < 0.05$; Fig. 4).

4. Discussion

The current study sought to examine the influence of the activation of 5-HT_{2A}Rs on hippocampal-dependent object memory, and its underlying physiological mechanisms. Our study yielded three main novel findings. First, we determined that 5-HT_{2A}Rs are distributed on the dendritic spines, shafts and presynaptic terminals found within the stratum radiatum of the CA1 region of the dorsal hippocampus of male C57BL/6J mice. Second, we found that a memory-enhancing systemic dose of the 5-HT_{2A}R agonist, TCB-2 significantly increased extracellular glutamate levels in dorsal hippocampus, and the mean firing rates of CA1 pyramidal neurons. Third, we observed that TCB-2 selectively enhanced object memory consolidation after systemic or intrahippocampal injection. In addition, we found that the memory-enhancing effect of TCB-2 was abolished by pretreatment with the 5-HT_{2A}R antagonist, MDL 11,939, indicating that the promnestic effect of the agonist is specific to 5-HT_{2A}Rs. To our knowledge, this is the first report to define, 1) the subcellular distribution of 5-HT_{2A}Rs in the CA1 region of dorsal hippocampus; and 2) that activation of 5-HT_{2A}Rs enhances the object memory consolidation, hippocampal glutamate release and CA1 neuronal firing.

Our electron microscopy data are the first to reveal a subcellular distribution of 5-HT_{2A}Rs on the presynaptic axon terminals, postsynaptic dendrites and dendritic spines of CA1 pyramidal neurons in the rodent dorsal hippocampus. These electron microscopy data are consistent with previous reports that 5-HT_{2A}Rs are expressed in the hippocampus (Luttgen et al., 2004), and are co-localized with NMDA- and AMPA-type glutamate receptors in the dentate gyrus (Peddie et al., 2008).

In the present study, we confirmed the enhancing effect of systemic TCB-2 on consolidation of object memory, and further demonstrated that the 5-HT_{2A}R agonist had no effect on the

encoding or retrieval, of object memory in mice. The enhancement of object memory, observed in mice that received post-training TCB-2, was dose-dependent and was prevented by MDL 11,939 pretreatment. The data provide further support for the conclusion that the object recognition enhancement is a consequence of the specific activation of 5-HT_{2A}Rs. Memory consolidation is a multi-faceted process that stabilizes the memory trace after its initial acquisition. Consolidation consists of a synaptic consolidation that occurs within the first minutes to hours after learning and a systems consolidation in which short-term memory is reorganized, enhanced, and may eventually become hippocampal-independent over a period of weeks to years (Dudai, 2004). The hippocampus is a critical structure for both object memory (Cohen et al., 2013; Hammond et al., 2004) and memory consolidation (Eichenbaum et al., 1999). Upon observing that activation of 5-HT_{2A}Rs selectively enhanced consolidation of object memory, we tested the influence of systemic TCB-2 on extracellular glutamate levels in dorsal hippocampus, and on the firing rates of CA1 neurons. The 5-HT_{2A}R has been found to directly interact with PSD-95 and regulate receptor trafficking and signal transduction (Xia et al., 2003). Glutamate neurotransmission and the density of AMPA/NMDA receptors in the postsynaptic zones are considered to influence synaptic plasticity and memory consolidation (Wang et al., 2006). Thus, our findings that 5-HT_{2A}Rs are present at dendritic spines of CA1 neurons and that activation of 5-HT_{2A}Rs enhances extracellular glutamate in hippocampus provides support for the view that 5-HT_{2A}Rs are positioned appropriately to modulate experience-triggered hippocampal glutamatergic-dependent synaptic plasticity. Indeed, the enhancement in object memory performance of mice that received intra-CA1 microinfusion of the 5-HT_{2A}R agonist is consistent with this model. Interestingly, Li et al. (2015) reported that intraseptal injection of TCB-2 increased firing rates of medial septal parvalbumin-positive neurons, and increased the

peak frequency of hippocampal theta rhythm in rats (Li et al., 2015). Thus, while we focused our analyses of TCB-2's effects on hippocampal physiological mechanisms, it appears that there are also extra-hippocampal sites of action at which systemic TCB-2 might enhance hippocampal function. Taken together, the findings reported here support the view that the memory enhancement observed after systemic TCB-2 is a consequence of the activation of hippocampal 5-HT_{2A}Rs.

In this study we also found that TCB-2 (1.0 mg/kg, i.p.) increased extracellular Glu levels in the CA1 region of the dorsal hippocampus. The principal neurons of the CA1 region are glutamatergic pyramidal neurons, which receive glutamatergic input from CA3 as well as direct input from other cortical regions, including entorhinal cortex. Glutamate sampled by microdialysis represents that which is synaptically released, carrier-mediated release, and that which results from glial metabolism. The non-synaptic glutamate measured *in vivo* include that which is released from glial cells, that which is derived from cystine–glutamate exchange, and (re)uptake via glutamate transporters. A significant portion of *in vivo* extracellular glutamate collected by microdialysis represents non-vesicular release, which is maintained predominantly by cysteine-glutamate exchangers expressed on neighboring glial cells (Baker et al., 2002).

Performance in the object recognition memory task is associated with an increase in hippocampal c-fos expression (Albasser et al., 2010) and glutamate efflux (Stanley et al., 2012). We previously demonstrated that novel object exploration is associated with an increase in glutamate efflux from the dorsal hippocampus (Cohen et al., 2013). The hippocampal CA1 region contains glutamatergic pyramidal neurons and receives glutamatergic input from CA3 and neocortex. The synaptic glutamate neurotransmission is critical for hippocampal memory processes. The present results suggest that TCB-2 may facilitate neuronal activity of distal

glutamatergic neurons that project to the CA1 region, for example pyramidal neurons in CA3 region, and/or CA1 pyramidal neuronal glutamatergic output into other brain regions (e.g., the subiculum) to facilitate object memory consolidation. Besides 5-HT_{2A}R-mediated neuronal activation, postsynaptic 5-HT_{2A}Rs may influence object memory consolidation by influencing NMDA receptor-mediated neurotransmission or plasticity. Activation of 5-HT_{2A}Rs produces an increase in the frequency and amplitude of cortical neuronal spontaneous excitatory postsynaptic potentials/currents (Aghajanian and Marek, 1999) and facilitates NMDA receptor activity and synaptic plasticity in the cortex (Arvanov et al., 1999) and basolateral amygdala (Chen et al., 2003); results which are consistent with our electrophysiological evidence that systemic TCB-2 treatment increases the mean firing rates of CA1 neurons recorded *in vivo*.

In vivo microdialysis and voltammetry using biosensors coated with glutamate oxidase are two classic methods to study extracellular levels of glutamate in the central nervous system. Due to technical restriction, neither of them can effectively distinguish between the synaptic pool and extrasynaptic pool of glutamate. The increase in glutamate efflux in response to TCB-2 treatment may represent a small glutamate pool of neuronal origin, which is only a fraction of the larger non-synaptic glutamate pool. Extracellular glutamate concentrations can be enhanced by a wide variety of pharmacological and physiological manipulations. However, it is presently impossible to confirm that the stimulated glutamate in dialysates are of neuronal origin (Timmerman and Westerink, 1997). The synaptic cleft is one sub-compartment where glutamate levels are tightly regulated by both synaptic and glial glutamate uptake, the influence of synaptic glutamate release is largely limited in space to the synaptic and immediate perisynaptic environment, thereby providing temporally coded excitatory neuronal communication in the msec range. In contrast, glutamate of glial origin in the non-synaptic extracellular compartment provides tonic influence

on synaptic release and postsynaptic responsiveness through pre- and post-synaptic mGluR (Moussawi et al., 2011). Extracellular glutamate of glial origin modulates glial and neuronal glutamate release and synaptic plasticity. Understanding how these glial and neuronal glutamatergic processes synergistically regulate extracellular glutamate concentrations is central to understanding the physiology of excitatory neuroplasticity and its emerging role in neuropsychiatric diseases.

We found that TCB-2 (1.0 mg/kg, i.p.) increased hippocampal glutamate efflux with a peak elevation observed 20-30 min post-injection and recovery to baseline levels by 50 min post-injection. We previously demonstrated that novel object exploration is associated with an increase in hippocampal glutamate efflux, that is, about a 20% increase from baseline (Cohen et al., 2013). This effect appears to be specific for novel object recognition, as novel context or environment exposure fails to alter hippocampal or cortical glutamate neurotransmission (Giovannini et al., 2001), suggesting that the elevation of hippocampal extracellular glutamate level may reflect the discriminative choice aspects (novel vs. familiar) of object exploration. We find that mice can successfully encode a memory for the novel object after having explored it for 30 s, which typically can be achieved within 5-10 min when they are allowed to freely explore the object in a familiar arena. However, the novel object exploration-induced increase in extracellular glutamate in dorsal hippocampus appears to be sustained for 30 min before returning to baseline. Considering that we find TCB-2 to selectively enhance object memory when administered post-sample session and not pre-sample, it is reasonable to suggest that post-sample TCB-2 may increase the exploration-induced glutamate efflux in the hippocampus at a time matched to the early phase of memory consolidation. Even though pre-sample TCB-2 would be expected to also increase hippocampal glutamate efflux, the timing of the glutamate

peak of this effect would be considerably earlier than that after post-sample TCB-2. Thus, the lack of effect of pre-sample TCB-2 on object memory may be a consequence of the drug-induced elevation of glutamate being out of phase with the task-induced elevation of hippocampal glutamate. Although, it will be difficult to precisely align the glutamate level with distinct memory processes, we have found TCB-2 to selectively influence object memory consolidation. It will be of interest to more precisely define the time window within which object memory consolidation remains sensitive to the activation of 5-HT_{2A}Rs. As mentioned above, the specific sources of glutamate and its neurobiological substrates to 5-HT_{2A}R activation are not fully understood. Glutamate sampled by microdialysis represents a balance of synaptic release, carrier-mediated release, and glial metabolism. To begin to distinguish the sources of the TCB-2-induced increase in extracellular glutamate will require combinatorial pharmacological studies involving transporter and antiporter blockers; experiments that are beyond the scope of the present work.

Some antipsychotic drugs, e.g. clozapine and olanzapine, are 5-HT_{2A}R inverse agonists; they act as antipsychotics and improve some, but not all, cognitive impairments in human (Rajagopal et al., 2013). Interestingly, systemic administration of 5-HT_{2A}R antagonists impairs spatial reversal learning with an operant paradigm (Boulougouris et al., 2008), but prevents the impairment of attention performance by NMDA receptor blockade in rats (Mirjana et al., 2004). These findings indicate that the modulatory effect of 5-HT_{2A}Rs on cognition depends on the species, behavioral paradigm, animal status, and other genetic and environmental profiles.

In summary, our findings indicate a phase-dependent influence of TCB-2 on hippocampal memory processes. Activation of 5-HT_{2A}R by systemic or intrahippocampal TCB-2 improved object memory consolidation, but did not affect encoding or retrieval. It is noteworthy that in rats,

the blockade of 5-HT_{2A}Rs in the prefrontal cortex impaired context-guided object memory, and memory for the temporal order in which objects were explored (Bekinschtein et al., 2013). We previously reported that administration of TCB-2 (1.0 mg/kg, i.p.) facilitated the acquisition of extinction of trace fear memory in mice, and that the post-conditioning administration of TCB-2 enhanced the consolidation of contextual and cued fear memory (Zhang et al., 2013). Results of our recent study suggest that pre-test administration of TCB-2 delays the retrieval of spatial memory in a Morris water maze task (Zhang et al., 2015). Taken together, it appears that 5-HT_{2A}R produces multi faceted modulation on memory and cognition, administration of TCB-2 immediately after a learning event enhances the consolidation of that memory, and such 5-HT_{2A}R-mediated enhancement may be related to increases in hippocampal glutamate efflux and neuronal firing. These results suggest that activation of 5-HT_{2A}Rs may represent a new neuropharmacological target for the treatment of memory impairments.

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Figure Legends

Fig. 1. Subcellular distribution of 5-HT_{2A}Rs in the pyramidal cell layer of the dorsal hippocampus

Electron micrographs of the CA1 region of the dorsal hippocampus showing immunoreactivity for 5-HT_{2A}Rs, as detected using a pre-embedding immunogold method. (A-E) Immunoparticles for 5-HT_{2A}R staining are mainly localized at postsynaptic compartments along the plasma membrane (arrows) and intracellular sites (crossed arrows) of dendritic spines (s) and dendritic shafts (Den) (arrows) of pyramidal cells. (F) Although less frequently, immunoparticles for 5-HT_{2A}Rs were also localized at presynaptic compartments (arrowheads) in axon terminals (at) establishing glutamatergic synapses with dendritic spines(s) of pyramidal cells. EM Scale bar: A-F, 200 nm.

Fig. 2. Systemic or hippocampal local infusion of TCB-2 enhances the consolidation of novel object memory

(A) Mice received TCB-2 before the sample session (TCB-2 + Vehicle + Vehicle, n=10), immediately after the sample session (Vehicle + TCB-2 + Vehicle, n=12) or before the test session (Vehicle + Vehicle + TCB-2, n = 10), presented 24 h later, to test object encoding, consolidation and retrieval, respectively. Mice treated with TCB-2 after the sample session demonstrated a stronger preference for the novel object during the test session than the vehicle-treated mice (Vehicle + Vehicle + Vehicle, n = 10), as measured by the discrimination ratio scores ($P < 0.05$). (B) Post-sample systemic TCB-2 dose-dependently enhanced object memory ($P < 0.01$). *Post-hoc* LSD tests indicated that mice administered 5 mg/kg TCB-2 exhibited a significantly greater discrimination ratio (n = 9, $P < 0.01$) than that of mice given 1 mg/kg TCB-2 (n = 12, $P < 0.05$), as compared to the vehicle-treated mice (n = 10). (C) MDL 11,939 (0.5

mg/kg, i.p.) was administered to mice immediately after the sample session and TCB-2 (1.0 mg/kg, i.p.) was given 10 min later. As in A and B, those mice that received post-sample TCB-2 exhibited enhanced performance during the test session, as evidenced by the higher discrimination ratio score ($n = 9$); however, this effect was blocked by MDL 11,939 pretreatment. There was no difference between the vehicle group ($n = 10$) and MDL 11,939 + TCB-2 group ($n = 9$). (D) Mice that received bilateral microinfusion of TCB-2 (1.0 μ g/0.5 μ l, $n = 8$) into the CA1 region exhibited enhanced performance during the test session as compared to mice that received intra-CA1 aCSF (0.5 μ l, $n = 7$). (E-F) illustrates the histologically identified location of each microinfusion within Cresyl violet-stained coronal sections of postmortem fixed brain tissue slices. Error bars are SEM. * $P < 0.05$, ** $P < 0.01$ vs. control.

Fig. 3. Activation of the 5-HT_{2A}R with TCB-2 increases extracellular glutamate levels in the CA1 region of dorsal hippocampus in freely moving male C57BL/6J mice

Systemic administration of TCB-2 (1.0 mg/kg, i.p.) induced (A) an increase in the extracellular level of glutamate in the dorsal hippocampus. The dashed horizontal line indicates the baseline extracellular glutamate levels and the arrow indicates the time of the systemic drug injection. (B) Histological identification of microdialysis probe locations within the hippocampus of Cresyl violet-stained coronal sections of mouse brain tissue sections. Data are expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. control. $n = 6-8$ per group.

Fig. 4. Activation of the 5-HT_{2A}R with TCB-2 increases neuronal firing in the CA1 region of dorsal hippocampus.

(A) *In vivo* neuronal activity was recorded from the CA1 region for 15 min after mice received a systemic injection of saline (0.9%, IP) and again after receiving TCB-2 (1 mg/kg, IP). Two additional mice received the injection of TCB-2 first and then received saline. There was no difference in the magnitude of effect on neuronal firing rates depending on order of systemic injection. Therefore, the figure includes all data combined. As illustrated the mean firing rate determined after TCB-2 treatment was significantly greater than that after saline, ($t(32) = -2.32$, $P < 0.05$). Data are expressed as mean $\pm$ SEM. * $P < 0.05$. (B) Histological identification of electrode locations within the dorsal hippocampus of Cresyl violet-stained coronal sections of mouse brain tissue sections. (C) Representative waveforms (top row) and clusters of spikes (bottom row) from a CA1 neuron recorded after the mouse received a saline injection (left), and again after the mouse received a TCB-2 injection (right).

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Figures

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