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Research report

# Inhibition of AMPAR endocytosis alleviates pentobarbital-induced spatial memory deficits and synaptic depression

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ABSTRACT

Our previous study has shown that pentobarbital causes memory deficits and impairs hippocampal synaptic plasticity. The Tat-GluA2$_{3Y}$ peptide (GluA2$_{3Y}$) prevents activity-dependent α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) endocytosis. It enables early-phase long-term potentiation (LTP) to proceed to late-phase LTP allowing short-term memory to convert to long-term memory. The purpose of this study is to explore the potential effects of GluA2$_{3Y}$ on pentobarbital-induced memory deficits through behavioral and electrophysiological paradigms. We found that *in vivo* intrahippocampal infusion of GluA2$_{3Y}$ (100 μM, 1 μl per hippocampus) 30 min prior to pentobarbital administration (8 mM, 1 μl per hippocampus) significantly rescued the pentobarbital-induced deficit of memory retrieval in rats during the Morris water maze test. Pre-incubation of GluA2$_{3Y}$ (10 μM) partially rescued bath application of pentobarbital-induced synaptic transmission of the CA3-CA1 pathway in hippocampal slices. More importantly, GluA2$_{3Y}$ selectively upregulated the synaptic GluA2 expression that was suppressed by pentobarbital. Together, these results suggest that inhibition of GluA2-containing AMPAR endocytosis by GluA2$_{3Y}$ increases the pentobarbital-suppressed basal synaptic transmission by upregulating the synaptic GluA2, and then subsequently alleviates spatial memory deficits. Therefore, inhibition of AMPAR endocytosis may be a potential therapeutic way to treat memory disorders caused by anesthetics.

## 1. Introduction

Pentobarbital, a barbiturate, has been used as a general anesthetic, to treat insomnia, for sedation and status epilepticus for many years. However, as an anesthetic drug, pentobarbital also has some side effects including amnesia, one of the important causes of postoperative cognitive dysfunction. Previous studies have shown that pentobarbital quantitatively impairs the memory acquisition process and is involved in short-term recall performance [1]. The systemic application of pentobarbital can produce memory dissociation in rats [2] and disrupt short-term memory and attention in monkeys when performing an operant behavioral test battery [3]. A recent study has reported that neonatal administration with pentobarbital leads to spatial memory impairment that can persist into adulthood [4]. We have previously shown that acute hippocampal microinjection of pentobarbital caused dramatic learning and memory deficits [5]. However, the mechanism of pentobarbital-induced memory deficit remains poorly understood.

It is widely accepted that synaptic plasticity in the CA1 area of the hippocampus, measured as long-term potentiation (LTP) and long-term depression (LTD), is the cellular mechanism underlying information processing and memory formation [6–8]. Evidence accumulated from recent studies suggests that LTD can be induced through a number of cellular processes that are either NMDA-dependent or NMDA-independent [9,10], with a common final step entailing the endocytosis of postsynaptic AMPARs [11,12]. Consistent with these findings, we have recently reported that inhibition of AMPAR endocytosis by a synthesized peptide GluA2$_{3Y}$ can prevent LTD expression [13–15] and prolong memory retention [16]. A previous study showed that administration of propofol, another highly effective intravenous anesthetic, produces dose-dependent suppression of LTP and basal synaptic transmission [17]. Similarly, our recent study revealed that pentobarbital suppressed hippocampal LTP and decreased neuronal excitability, which may lead to spatial learning and memory deficits [5]. Therefore, we hypothesized that inhibiting AMPAR endocytosis by GluA2$_{3Y}$ may alleviate the pentobarbital-induced synaptic and memory deficits. In the present study, we investigated this hypothesis by using a combination of electrophysiological, behavioral and biochemical assessments.

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## 2. Materials and methods

### 2.1. Animals

Male Sprague-Dawley rats weighing 200–250 g were obtained from Chongqing Medical University Animal Care Center and housed in the laboratory colony of the Children's Hospital of Chongqing Medical University. The rats were given free access to food and water, and housed in a room that was maintained at 21 °C (on a 12-h dark/light cycle). All procedures were performed in accordance with Chongqing Science and Technology Commission guidelines for animal research and approved by the Chongqing Medical University Animal Care Committee. All efforts were made to minimize the number of animals used.

### 2.2. Reagents

Pentobarbital was purchased from Sigma-Aldrich. The AMPAR endocytosis inhibitor $GluA2_{3Y}$ ($_{869}$YKEGYNVYG$_{877}$ in the carboxyl-terminal region of the AMPA GluA2 subunit) and scrambled control $GluA2_{3Y}$ peptide (Scr-$GluA2_{3Y}$, $_{869}$VYKYGGYNE$_{877}$) were synthesized by GL Biochem Ltd (Shanghai, China). For intrahippocampal injection, the pentobarbital was dissolved in 0.9% sterile saline to 8.0 mM, $GluA2_{3Y}$ and Scr-$GluA2_{3Y}$ were dissolved to 100 μM. For *in vitro* treatment, all the reagents were dissolved in the artificial cerebrospinal fluid (ACSF) at different concentrations.

Anti-PSD 95 antibody were obtained from Millipore. Anti-GluA1, anti-GluA2 and anti-β-actin antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA).

### 2.3. Bilateral hippocampal microinjection

For intrahippocampal injection, the rats were first implanted with a cannula into the dorsal hippocampus as previously described [14,16]. Briefly, under isoflurane anesthesia, a rat was placed in a stereotaxic apparatus (Stoelting, USA) and implanted with two 22-gauge stainless steel guide cannula (10 mm; Plastics One Inc., Roanoke, VA) above the dorsal hippocampus (3.5 mm posterior to the bregma, 2.5 mm lateral to the midline and 2.5 mm below the surface of the dura). The guide cannula was fixed to the skull with four jeweler's screws and dental cement. A sterile dummy cannula (30-gauge stainless steel rod, 10 mm, Plastics One Inc.) was inserted into the guide cannula to prevent bacterial infection and leakage of cerebral spinal fluid through the cannula. After surgery, all animals were allowed a 7-day post-operative recovery period before the behavioral experiments.

On the day before behavioral experiments, the animals were given a sham intra-hippocampal injection to acclimatize them to the injection procedure. Each rat was kept in an empty Plexiglas box (25 × 45 × 25 cm). The dummy cannula was then replaced by an injection cannula (30-gauge) which was connected to a microsyringe pump (Harvard Apparatus) via PE-50 tubing. The drugs were injected by using a 10-μl Hamilton syringes at the rate of 0.5 μl/min for 2 min. The injection cannula was kept in place for an additional minute for the drug diffusion. The dummy cannula was placed back, and the rats were moved back into their home cages. The injection position was verified by histological examinations of the brains after methylene blue injection (1 μl per side). Only the data obtained from the rats with correctly inserted cannula were included in statistical analyses.

### 2.4. Morris water maze test

Spatial learning and memory were examined with the Morris water maze using procedures similar to those described previously [13,16]. The Morris water maze was a circular fiberglass pool (180-cm diameter), filled with water (25 ± 1 °C) and artificially divided into four quadrants, i.e., N, E, S, and W. Black non-toxic paint was used to make the water opaque. A light blue curtain with fixed distal visual cues surrounded the pool. The video of each test was recorded and analyzed by ANY-maze tracking system (Stoelting, USA).

Twenty-four hours before the spatial training, the animals were allowed to swim for 60 s to adapt to the maze. They were then trained for four trials per day for five consecutive days. To determine the effect of $GluA2_{3Y}$ on pentobarbital-impaired spatial memory, the rats were pre-injected with $GluA2_{3Y}$ ($GluA2_{3Y}$ + pentobarbital) or Scr-$GluA2_{3Y}$ (Scr-$GluA2_{3Y}$ + pentobarbital) for 30 mins before pentobarbital injection. The control was injected with saline only. Each rat took the maze probe test with the platform removed 20 min after the last injection.

### 2.5. Electrophysiology

The rats were deeply anesthetized using urethane (1.5 g/kg, i.p.) and transcardially perfused with ice-cold artificial cerebral spinal fluid (ACSF) prior to decapitation as described previously [18]. The hippocampus was removed and acute coronal slices were sectioned (400 μm thick) with a vibratome (VT1000S, Leica Microsystems) in ACSF bubbled with 95% $O_2$ and 5% $CO_2$. The slices were then incubated in oxygenated ACSF for 1 h at 30 °C. The field excitatory postsynaptic potentials (fEPSPs), evoked by stimulation of the Schaffer collateral/commissural pathways, were recorded in the CA1 area of the hippocampus using pipettes (3–4 MΩ) filled with ACSF. Pentobarbital, $GluA2_{3Y}$ and Scr-$GluA2_{3Y}$ were dissolved in ACSF at the required concentration. Data acquisition (filtered at 3 kHz and digitized at 10 kHz) was performed with the PatchMaster v2.73 software (HEKA Electronic, Lambrecht/Pfalz, Germany).

### 2.6. Western blotting

Immediately after the Morris water maze test, the hippocampus was collected and homogenized in the ice-cold Tris-HCl buffer, then centrifuged twice at 4 °C at 700*g* for 7 min. The 2 supernatants were pooled and centrifuged at 100,000*g* at 4 °C for 60 min. Pellets were re-suspended in the buffer containing 0.5% Triton X-100 and incubated at 4 °C for 20 min. Then layered over 1 M sucrose, and centrifuged at 100,000*g* for 60 min. The Triton-insoluble material that sedimented through the sucrose layer was re-suspended in the buffer containing 1% SDS and stored at −80 °C (mainly postsynaptic densities). Total protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Samples were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes. After incubated with 5% fat-free milk for 1 h at room temperature, the target proteins were immunoblotted with primary antibody overnight at 4 °C to GluA1 (1: 500) and GluA2 (1: 1000) followed by incubation with secondary antibody (1: 3000, 1 h at room temperature). The blots were visualized in the Bio-Rad Imager. PSD-95 (1: 500) and β-actin (1: 3000) were used as the postsynaptic and cytoplasmic marker separately.

### 2.7. Statistical analyses

All data were presented as mean ± SEM. The data were analyzed with a one-way ANOVA followed by post hoc Turkey's test, with treatment (group) as the between-subjects factor. The significance level was set at $p < 0.05$.

## 3. Results

### 3.1. Inhibition of AMPAR endocytosis significantly relieves pentobarbital-induced spatial memory deficits

Our previous study has shown that a single pentobarbital treatment (20 min before the probe test in the Morris water maze paradigm) impairs the memory retrieval [5], and inhibiting AMPAR endocytosis