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Naltrexone Facilitates Learning and Delays Extinction by Increasing AMPA Receptor Phosphorylation and Membrane Insertion

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ABSTRACT

BACKGROUND: The opioid antagonists naloxone/naltrexone are involved in improving learning and memory, but their cellular and molecular mechanisms remain unknown. We investigated the effect of naloxone/naltrexone on hippocampal α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) trafficking, a molecular substrate of learning and memory, as a probable mechanism for the antagonists activity.

METHODS: To measure naloxone/naltrexone-regulated AMPAR trafficking, pHluorin-GluA1 imaging and biochemical analyses were performed on primary hippocampal neurons. To establish the in vivo role of GluA1-Serine 845 (S845) phosphorylation on the behavioral effect induced by inhibition of the endogenous μ-opioid receptor (MOR) by naltrexone, MOR knockout, and GluA1-S845A mutant (in which Ser^{845} was mutated to Ala) mice were tested in a water maze after chronic naltrexone administration. Behavioral responses and GluA1 levels in the hippocampal postsynaptic density in wild-type and GluA1-S845A mutant mice were compared using western blot analysis.

RESULTS: In vitro prolonged naloxone/naltrexone exposure significantly increased synaptic and extrasynaptic GluA1 membrane expression as well as GluA1-S845 phosphorylation. In the MOR knockout and GluA1-S845A mutant mice, naltrexone did not improve learning, which suggests that naltrexone acts via inhibition of endogenous MOR action and alteration of GluA1 phosphorylation. Naltrexone-treated wild-type mice had significantly increased phosphorylated GluA1-S845 and GluA1 levels in their hippocampal postsynaptic density on the third day of acquisition, which is the time when naltrexone significantly improved learning.

CONCLUSIONS: The beneficial effect of naltrexone on spatial learning and memory under normal conditions appears to be the result of increasing GluA1-S845 phosphorylation-dependent AMPAR trafficking. These results can be further explored in a mouse model of memory loss.

Keywords: AMPA receptors, GluA1, GluA1-S845, GluA1-S845A mutant, Naltrexone, Spatial memory

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The endogenous opioid system modulates many functions, including learning and memory. For instance, intracerebral administration of ß-endorphin or endomorphin-1/2 in rodents significantly impairs retention memory and learning in both inhibitory/passive avoidance and water maze tasks (1,2). Also, the μ-opioid receptor (MOR) agonist morphine, an exogenous opioid that mimics endorphin action, modulates learning and memory in both humans and animals (3,4), which is usually counteracted by an opioid antagonist, such as naloxone or naltrexone. More importantly, other investigations demonstrated that posttraining administration of naloxone or naltrexone alone, which presumably inhibits endogenous opioids, ameliorates the retention of both passive and active avoidance conditioning (5–7) as well as acquisition on a radial arm maze in a novel spatial environment in rats (8). Moreover, experiments conducted on monkeys showed a significant naloxone-induced improvement of memory in animals with especially poor control scores, supporting the possibility that individuals with weak memory may be particularly responsive to the facilitating effects of naloxone/naltrexone (9). However, the molecular and cellular mechanisms by which naloxone/naltrexone promotes enhanced memory still remain unknown.

One of the neurobiological modifications underlying different forms of learning has been shown to involve the recruitment and activation of additional functional excitatory glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) on the neuronal postsynaptic membrane and increasing of dendritic spine size and density (10–12). GluA1, one of the four subunits constituting ionotropic AMPAR (13), plays a key role in activity-dependent synaptic trafficking of AMPARs, especially in the adult hippocampus, which is, among others, an essential structure for spatial learning and memory (14).

Chronic administration of morphine decreases the density of dendritic spines in vitro (15–17) and in vivo (18), as well as the amount of synaptic GluA1 through internalization in cultured hippocampal neurons (19). Also, in a behavioral model of drug addiction, morphine can induce context-dependent

sensitization through changes of AMPAR expression and phosphorylation in the postsynaptic density (PSD) of mice hippocampi (20). In contrast, treatment with naloxone or naltrexone or genetic deletion of MOR increases the density of dendritic spines (15). Thus, a probable mechanism by which naloxone and naltrexone improve learning and memory might be by affecting AMPAR trafficking through MOR inhibition.

In the present study, we first established that the facilitative effects of chronic naltrexone treatment on acquisition and retention in a water maze task are mediated by the blockade of endogenous MOR. Using cultured hippocampal neurons and following treatment with naloxone and naltrexone, we then observed induction of the temporal translocation of GluA1 from the intracellular compartment to the surface membrane with a parallel increase in GluA1-Serine 845 (S845) phosphorylation, which has been shown to affect AMPAR trafficking and synaptic delivery (21,22). The recruitment of AMPARs on the membrane surface and GluA1-S845 phosphorylation may be related to the memory improvements observed during acquisition, which was demonstrated by comparing the behavioral responses and GluA1 levels in the PSD in both wild-type and GluA1-S845A mutant mice with chronic naltrexone administration.

METHODS AND MATERIALS

Animals

Heterozygous GluA1-S845A mutant and homozygous C57BL/6J MOR knockout (MORKO) mice were generous gifts from Hey-Kyoung Lee, Ph.D., (Johns Hopkins School of Medicine, Baltimore, Maryland) and John Pintar, Ph.D., (Robert Wood Johnson Medical School, Piscataway, New Jersey), respectively. The homozygous mice in which the *Cre* gene was absent were produced from the heterozygous GluA1-S845A line and identified by polymerase chain reaction. Wild-type, homozygous GluA1-S845A and MORKO mice were used for breeding and housed in a temperature-controlled (21°–23°C) environment with a 12-hour light/dark cycle. Food and water were provided ad libitum. Experiments were conducted on 2-month-old male mice (30–35 g). All animal procedures followed the National Institutes of Health guidelines and were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Drug Treatment

Naltrexone hydrochloride was dissolved in .9% sodium chloride and was injected intraperitoneally at a concentration of 2 mg/kg twice per day. The mice were tested 30 minutes after injection.

Morris Water Maze

The task was carried out as previously described (23).

For the acquisition of spatial reference memory from days 1 to 6, each mouse was given four trials of a maximum 60 seconds per day to find the hidden platform placed in the middle of the southwest quadrant. To initiate the trials, each animal was placed at randomized positions in the tank. During intertrial intervals of 60 seconds, each mouse was allowed to stay on the platform until the next trial. The latency to find and climb on the platform, as well as the swimming distance and speed, were recorded and averaged daily.

Retrieval of reference memory was performed on day 7, 24 hours after the last training trial. Mice were given a 30-second retention (probe) test with the platform removed. The swim path and time spent in the platform (training) quadrant were recorded over 30 seconds. To test the extinction of the quadrant preference, interspersed probe trials were conducted, besides the day 7, on days 10 and 13 (4 and 7 days after the last training trial, respectively). See Supplemental Methods and Materials for a detailed description.

A video-computerized tracking system (ANY-maze, Stoelting Co., Wood Dale, Illinois) was used to record and analyze animal behavior.

Transfection of Hippocampal Cultures and Live-Cell Confocal Imaging

Dissociated neuronal cultures were prepared from the hippocampi of Sprague-Dawley rats (Harlan Laboratories, Indianapolis, Indiana) and C57BL/6J mice (Charles River Laboratories, Wilmington, Massachusetts) on postnatal days 1 and 2 and maintained as previously described (24,25). For live-cell imaging experiments, neurons were plated at a density of 1×10^6 cells per dish onto a 35-mm glass bottom Petri dish coated with poly-L-lysine (thickness of glass coverslip, .08 mm) (26). For all other biochemical studies, 3×10^6 cells were plated onto a poly-L-lysine treated 60-mm Petri dish. From the day of plating, cultured neurons were counted as day 1 in vitro (DIV1). Neurons on DIV5 to DIV9 were transfected with pHluorin-GluA1 (19) using a standard calcium phosphate co-precipitation method and were used for experiments between DIV18 and DIV24. The averaged pHluorin-GluA1 fluorescence of the cells was based on the quantification of the pHluorin-GluA1 intensity of each individual neuron examined under a Leica DMIRE2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) connected to a BD CARVII confocal imager (BD Biosciences, San Jose, California) and a Hamamatsu EM CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). See detailed description in Kam *et al.* (19) and Supplemental Methods and Materials.

Surface Biotinylation on Hippocampal Cultured Neurons

After incubation with or without 10 μmol/L naloxone or naltrexone for 1 day, the surface of cultured mouse and rat neurons (DIV18) were biotinylated according to the manufacturer's instructions (Pierce Biotechnology, Rockford, Illinois). Fifty microliters of each biotinylated sample were used to determine the total GluA1 input, and equal amounts of the remaining samples were immunoprecipitated and used for sodium dodecyl sulfate polyacrylamide gel electrophoresis/western blot analysis.

Subcellular Fractionation and Western Blot Analysis

The protocol used to isolate PSDs from the hippocampi was a modification of previously described procedures (27,28). Hippocampi from six mice were pooled, resuspended in a solution