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

Supplemental Information

Contents

Supplemental Methods and Materials

Morris Water Maze (detailed)

The procedure was carried out as previously described in (1). Mice were trained in a 120-cm diameter circular white maze purchased from Stoelting Co (Wood Dale, Illinois) and filled with opaque water (19-22°C). Four spaced points around the edge of the pool were used as starting positions: east, southeast, northwest and north. A circular platform of 10-12 cm diameter was placed in the center of the southwest quadrant. The maze was surrounded by immovable extra-maze distal visual cues. During the experiment, mice were required to locate the hidden submerged platform (0.5-1 cm below the surface), which remained at the same position across all trials for individual animals. A video-computerized tracking system (ANY-maze, Stoelting Co, Wood Dale, Illinois) was used to record and analyze the animal's behavior.

Acquisition of Spatial Reference Memory. During the acquisition from Days 1 to 6, each mouse was given 4 trials of 60 seconds per day (mice were placed at different starting positions in the tank to initiate each trial). The latency to find the escape platform as well as the swimming distance and speed were recorded and averaged daily. After finding and climbing onto the platform, the mice were allowed to stay on the platform for 60 seconds until the next trial.

Retrieval/Extinction. On Day 7, 24 hours after the last trial, mice were given a retention (probe) test. The platform was removed, and the swim path and time spent in the platform ("training") quadrant were recorded over 30 seconds. The animal was placed in a different starting position in the maze (northeast) facing the tank wall which was 180°C from the original platform position. To test the extinction of the quadrant preference, interspersed probe trials were conducted on Day 7 as well as on Days 10 and 13 (4 and 7 days after the last training trial, respectively).

Cued Learning. To determine the differences in the sensorimotor function between mouse lines, a cued learning task with a visible platform was administered. Curtains were closed around the maze to remove the availability of distal cues. The platform was tagged by a flag. The task consisted of 6 days training with 4 trials/day and 120 seconds/trial. All mice had 30 seconds inter-trial intervals on the platform and 30 seconds in a holding cage while the platform was moved to its new location. Platform and start positions were randomized over the trials.

Working Memory. The acquisition of working memory involved trial-dependent (matching-to-sample) learning of the platform location. The daily spatial working task contained a location or sample trial (first trial) and 3 matching trials (second, third and fourth trials) separated by 20-seconds intervals. Both the starting and platform positions were changed daily for every set of 4 trials.

Barnes Maze – Cued Learning

The Barnes maze is a hippocampal-dependent spatial learning and memory task which uses as the motivational drive, the rodents instinctive aversion for brightly lit, exposed circular open surface to escape into a small dark recessed chamber located under one of the 20 holes around the perimeter of the platform. The protocol followed has been previously described in (2). The maze consisted of a non-reflective gray, circular platform (90 cm diameter) that was elevated 90 cm above the floor, with 20 equally spaced with a gray escape box/tunnel ($20 \times 5 \times 6$ cm^3) placed under one of the holes (Stoelting Co, Wood Dale, Illinois). The center of the maze was illuminated by a bright light.

In the cued-target control version of the task, the maze was surrounded by a black curtain to block distal visual cues and the escape/target hole was designated by a flag with painted high

contrast vertical stripes. Over the course of the trials, the mice learned to use the flag to locate the escape hole. During the period of habituation, before the first day of testing, the mice were placed in a cylindrical black start chamber in the middle of the maze. After 10 seconds the start chamber was removed, a buzzer and light were turned on and the mice were gently guided to the escape box where it would stay for 2 minutes. Once the mouse was inside the box, buzzer and light were turned off. The mice received 6 consecutive days of cued training consisting of 4 trials per day, with an inter-trial interval of approximately 15 minutes. The trial would start by placing the mouse in the middle of the platform under a black cylinder. After 10 seconds, the start chamber was removed, the buzzer and light were turned on, and the mouse was set free to explore the maze until it entered the escape tunnel or after 3 minutes elapsed. Once in the escape box, the mouse was allowed to stay in the dark for 1 minute. If the mouse does not reach the goal within 3 minutes, the experimenter would guide the mouse to the escape box. Then, the animal was placed in its home cage until the next trial. The location of the target hole was changed between the trials. The measures recorded in each session included the total latency and distance to enter the escape box with all four paws and total number of errors (nose pokes and head deflections over any hole that did not have the tunnel beneath it) made before entering the escape hole. However, despite having learned the association between the intra-maze cue and the escape location, some mice continued to make errors by further exploring the maze. Thus the measure of the total latency, distance and errors may not accurately reflect the mouse learning. That is why we calculated the latency, distance and number of errors to the first encounter of the escape hole, called primary latency, primary distance and primary errors.

Live-Cell Confocal Imaging and Data Analysis (detailed)

Images were acquired using a 63× oil-immersion objective lens on a Leica DMIRE2 fluorescence microscope connected to a BD CARVII confocal imager and a Hamamatsu EM CCD camera (3). To maintain neuronal viability during image capturing, transfected hippocampal cultures were placed in a temperature-controlled chamber installed on the microscope (heated at 37°C) under a 5% CO_2 atmosphere (Leica Microsystems, Buffalo Grove, Illinois). Cells were then immediately returned to a humidified 5% CO_2 incubator at 37°C for prolonged drug treatment. All transfected cells, except those with abnormal morphologies, were randomly photographed. The *x*,*y* coordinates of individual neurons on the stage fitted with *x*,*y* translation were recorded by the IPLab4 software program (BDBiosciences, San Jose, California) so that the same neuron could be located and imaged at different time points during the drug treatment. All confocal images were taken as stacks ($\leq$ 25 *z* planes) at 0.4 μm intervals and then merged into a single image before further analysis with IPLab4 software.

The averaged pHluorin-GluA1 fluorescence of the cells was based on the quantification of the pHluorin-GluA1 intensity of each individual neuron in the field of view. To obtain these data, total area of the cell from the entire image was selected by an autosegmentation function in the IPLab4 program. In the case of measuring averaged pHluorin-GluA1 fluorescence on specific regions such as spines and dendrites, the region of interest was manually highlighted with Segment Tools. All fluorescence measurements represented raw data with background subtractions of the averaged blank field intensities. All data are expressed as mean ± SEM of the indicated number of experiments. Statistical significance was determined using *t* test for a comparison between two groups and ANOVAs for a comparison between multiple groups. A value of $p < .05$ was considered statistically significant.

Subcellular Fractionation and Western Blot Analysis (detailed)

The protocol used to isolate PSDs from the hippocampi was a modification of previously described procedures (4,5). Hippocampi from 6 mice were pooled, resuspended in a solution containing 0.32 M sucrose, 4 mM HEPES (pH7.4) with protease/phosphatase inhibitors (Sigma-Aldrich, St. Louis, Missouri) and homogenized using 15 strokes of a motor-driven glass-Teflon homogenizer. The resultant homogenates were centrifuged at 1000 × *g* at 4°C for 10 minutes to obtain the supernatant (S1). S1 was centrifuged at 10000 × *g* for 15 minutes to yield the crude synaptosomal pellet (P2) which was washed in 10 volumes of HEPES-sucrose homogenization buffer and centrifuged at 10000 × *g* for another 15 minutes. The washed sample P2 was lysed by hypo-osmotic shock in 9 volumes of ice-cold *d*H_2O with protease/phosphatase inhibitors, followed by 3 strokes of a motor-driven glass-Teflon homogenizer. Then, after adjusting the concentration to 4 mM HEPES using a 1-M HEPES, pH 7.4 stock solution and rotating for 30 minutes at 4°C, lysing was complete. The resulting lysate P2 was centrifuged at 25000 × *g* for 20 minutes to obtain the crude synaptic vesicle fraction supernatant (S3) and lysed synaptosomal membrane fraction pellet (P3), which was resuspended in 3 mL homogenization buffer. The sucrose gradient, which contained 3 mL of the resuspended P3 and 3 mL each of 0.8, 1 and 1.2 M sucrose solutions all containing protease/phosphatase inhibitors, was then centrifuged at 150000 × *g* for 2 hours. The PSD band between 1 M and 1.2 M sucrose was removed and diluted to 0.32 M sucrose by adding 2.5 volumes of 4 mM HEPES pH 7.4 with protease/phosphatase inhibitors. This solution was centrifuged at 150000 × *g* for 20 minutes, and the pellet was resuspended in 50 mM HEPES, 2 mM EDTA pH 7.4 with protease/phosphatase inhibitors and TritonX-100 (0.5% final). After rotating at 4°C for 15 minutes, the resuspended pellet was centrifuged at 22000 × rpm for 30 minutes to obtain PSD pellet, which was resuspended in 50

mM HEPES and 2 mM EDTA pH 7.4 with protease/phosphatase inhibitors. The proteins were then separated by SDS-PAGE and immunoblotted with antibodies against actin, phospho-GluR1-S845 (Millipore, Massachusetts) and GluR1 (Santa Cruz Biotechnology, California).

Supplemental Results

Effect of Chronic 6β-Naltrexol Administration on the Acquisition of Spatial Reference Memory in Wild-type Mice

Because some studies suggested that naloxone and naltrexone act as inverse agonists under certain conditions (e.g. after morphine pretreatment, but not in drug-naïve animals) (6,7), we cannot clearly attribute the behavioral effect of the two drugs to a blockade of the endogenous opioids actions on MOR. That is why we assessed the chronic effect of a well-known MOR neutral antagonist, 6β-naltrexol (6,7), on the acquisition of spatial memory with the water maze. Wild-type mice were given twice-daily intraperitoneal injections of 6β-naltrexol, at equipotent dose of 20 mg/kg for 6 days. Two-way ANOVAs on escape latency and distance revealed significant main effects for "training day" (Figure S1B: latency $F_{1,46} = 10.55$, $p < .01$; Figure S1C: distance $F_{1,46} = 6.68$, $p < .05$), "treatment" (Figure S1B: latency $F_{5,230} = 87.69$, $p < .001$; Figure S1C: distance $F_{5,230} = 65.06$, $p < .001$) and the interaction "treatment × training day" (Figure S1B: latency $F_{5,230} = 2.26$, $p < .05$; Figure S1C: distance $F_{5,230} = 2.29$, $p < .05$). Treatment with 6β-naltrexol facilitated learning as shown by shorter time and distance to find the hidden platform in 6β-naltrexol-treated wild-type mice compared to the vehicle-treated mice from Day 2 to Day 4. Post hoc analyses confirmed significant differences in escape latency and distance between the drug-treated and control groups on Day 2 (Figures S1B and S1C: latency and distance $p < .001$). Treatment with the neutral antagonist 6β-naltrexol induces a faster learning in the wild-type mice and these results were similar to those measured with naltrexone. Thus, we can suggest that naltrexone acts as a neutral antagonist to induce its learning improvement in the wild-type mice.

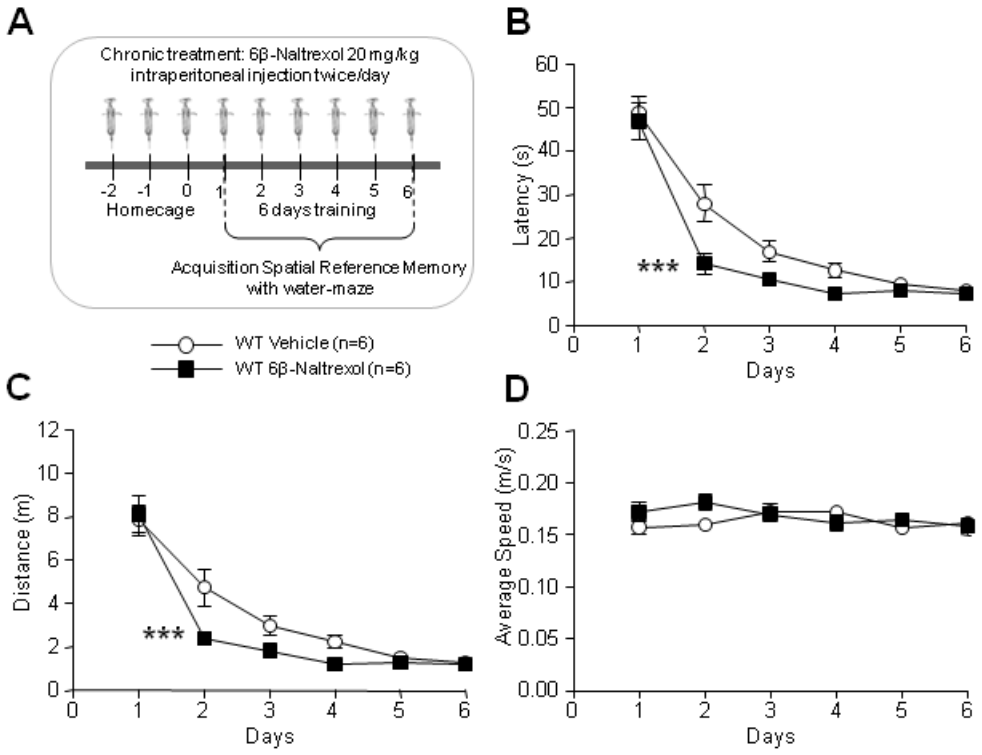

Figure S1. Effect of chronic 6β-naltrexol administration on the acquisition **(A-D)** of spatial reference memory in wild-type mice in a water maze task. Points and whiskers are means ± SEMs. Chronic intraperitoneal treatment with 6β-naltrexol 20 mg/kg twice/day started 3 days (-2, -1, 0 on the timeline) before the first day of the training period of the task during which the mice were given 4 trials/day to find the hidden platform for 6 consecutive days (1 to 6 on the timeline) **(A)**. The 6β-naltrexol-treated wild-type mice (filled squares, $n = 6$) required less time **(B)** and distance **(C)** to find the submerged platform compared to vehicle-treated wild-type mice (open circles, $n = 6$). **(D)** There were no significant differences in the average swimming speed among the different groups during the 6 days of acquisition ($n = 6$/group). Significant differences among groups were determined using two-way ANOVAs, followed by Newman-Keuls's post hoc comparisons. *** $p < .001$ significant differences between vehicle- and 6β-naltrexol-treated wild-type mice.

Cued Learning in Wild-type and GluA1-S845A Mutant Mice in the Water Maze

A cued platform task with wild-type and GluA1-S845A mutant mice was performed to determine whether differences in non spatial learning (association visual cue/hidden platform), escape motivation and visuomotor capabilities, exist (Figures S2A-D). No differences were noted for the main effect of "genotype" or any interaction with "genotype" when comparing the latency, distance and average speed of saline-treated wild-type and GluA1-S845A mutant mice. Also, naltrexone treatment does not affect the cued performances in the two mouse lines in the water maze (Figures S2B-D) as well as on the Barnes maze (Figures S3A-G).

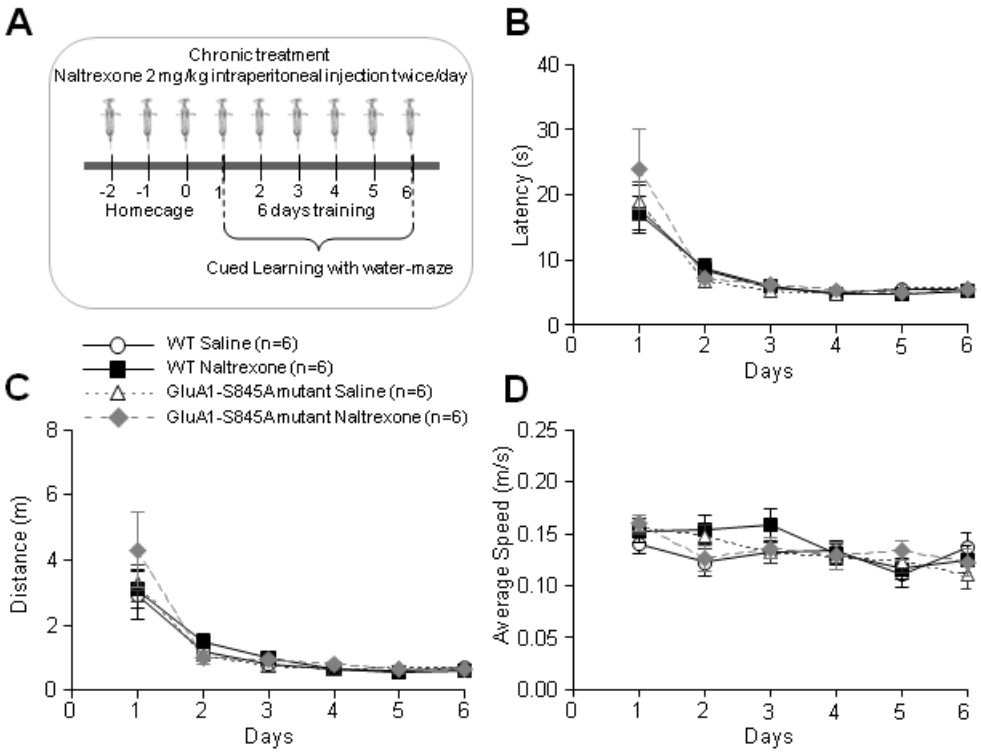

Figure S2. Effect of chronic naltrexone administration on the cued version of the water maze task in GluA1-S845A mutants and wild-type littermates **(A-D)**. Points and whiskers are means ± SEMs. Chronic intraperitoneal treatment with naltrexone 2 mg/kg twice/day started 3 days (-2, -1, 0 on the timeline) before the first day of the training during which the mice were given 4 trials/day to find the visible platform for 6 consecutive days (1 to 6 on the timeline) **(A)**. All mice learned to find the cued platform during the 6 training days. There were no significant group (n = 6/group) differences on the measures of latency **(B)**, distance **(C)** and average speed **(D)**.

Cued Learning in Wild-type and GluA1-S845A Mutant Mice on the Barnes Maze

A cued-target control version of the Barnes maze where the escape hole was marked with a visible proximal cue was carried out to determine whether there are differences in non spatial learning (association visual cue/hidden platform), escape motivation and sensorimotor abilities between the wild-type and GluA1-S845A mutants mice (Figures S3A-G). As shown on Figure S3, learning was apparent across all groups, saline- and naltrexone-treated wild-type mice as well as GluA1-S845A mutants, as observed by shorter latencies and distances and fewer errors to reach the escape hole across the days. Consistent with these observations, a three-way ANOVA showed a significant main effect of "training day" [(Figure S3B: $F_{5,520} = 221.23$, $p < .001$; Figure S3C: $F_{5,520} = 52.39$, $p < .001$), (Figure S3D: $F_{5,520} = 380.69$, $p < .001$; Figure S3E: $F_{5,520} = 51.799$, $p < .001$), (Figure S3F: $F_{5,520} = 128.28$, $p < .001$; Figure S3G: $F_{5,520} = 34.13$, $p < .001$)]. However, no significant differences were observed for the main effect of "genotype" or any interaction with "genotype" when comparing the total/primary latency, distance and errors between the saline-treated wild-type and GluA1-S845A mutant mice (Figures S3B-G). This indicates that the GluA1-S845A mutation does not seem to affect the sensorimotor function, associative learning or motivation in the cued version of the Barnes maze. Also, naltrexone treatment at a dose of 2 mg/kg twice a day does not induce significant changes in the cued performances in the two lines of mice with the same behavioral task.

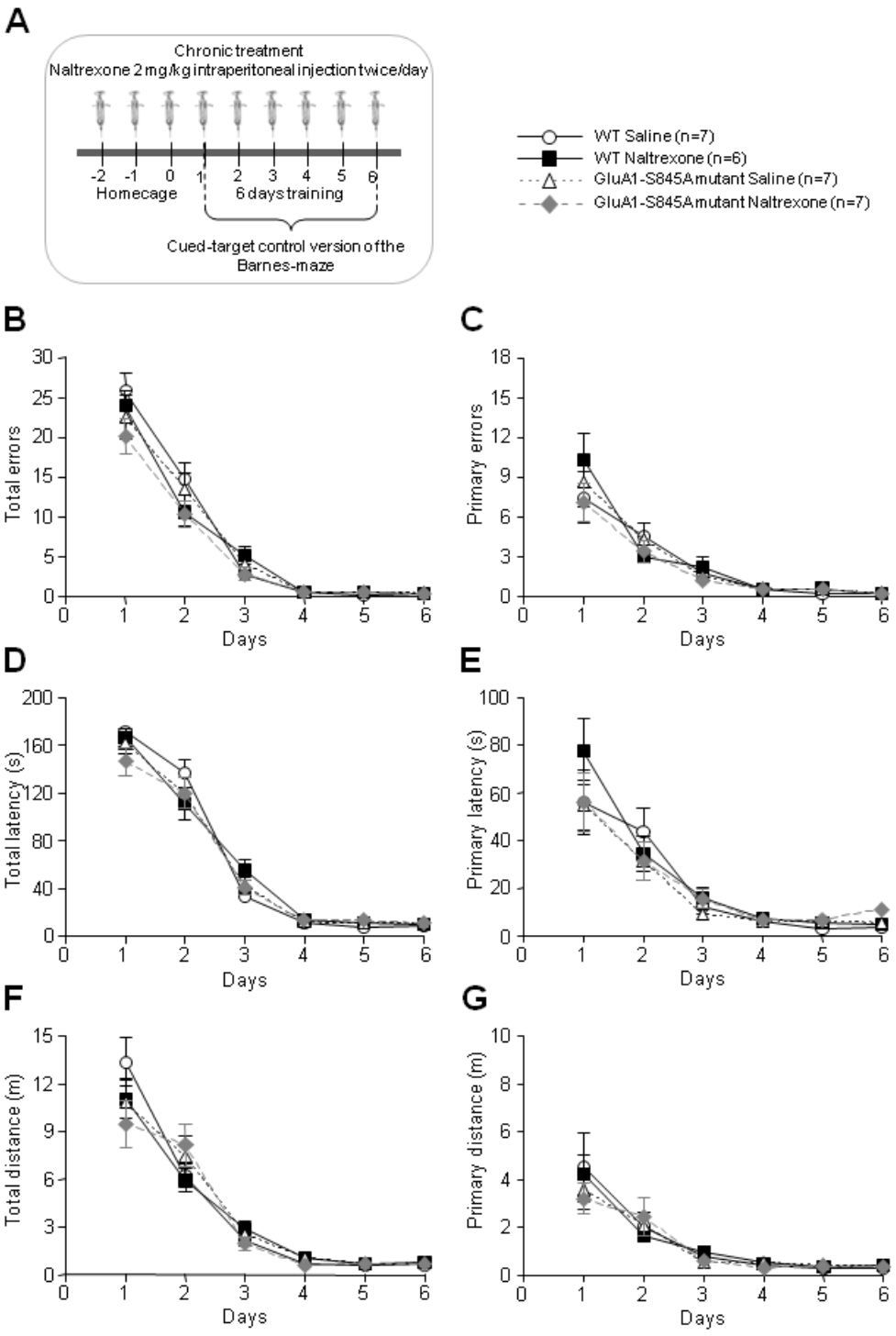

Figure S3. Cued learning in GluA1-S845A mutants and wild-type littermates on the Barnes maze **(A-G)**. Points and whiskers are means ± SEMs. Chronic intraperitoneal treatment with naltrexone 2 mg/kg twice/day started 3 days (-2, -1, 0 on the timeline) before the first day of the training period of the task during which the mice were given 4 trials/day to find the cued escape hole for 6 consecutive days (1 to 6 on the timeline) **(A)**. Four groups of mice were tested: saline-

treated (open circles, $n = 7$) and naltrexone-treated (filled squares, $n = 6$) wild-type mice, saline-treated (open triangles, $n = 7$) and naltrexone-treated (filled diamonds, $n = 7$) GluA1-S845A mutants. There were no significant group differences on the measures of total errors **(B)**, primary errors **(C)**, total latency **(D)**, primary latency **(E)**, total distance **(F)** and primary distance **(G)** during the 6 days of cued acquisition.

Effect of Chronic Naltrexone Injections on the Acquisition of Spatial Working Memory in Wild-type and GluA1-S845A Mutant Mice

Since naloxone/naltrexone are known to enhance working memory (8,9), we tested the role of GluA1-S845 phosphorylation in naltrexone's effects on working memory with the GluA1-S845A mutants in the water maze (Figures S4A-F). We observed significant effects of "treatment" (latency $F_{1,36} = 12.21$, $p < .001$; distance $F_{1,36} = 8.7$, $p < .01$), "treatment × genotype × trial" (latency only $F_{3,108} = 3.22$, $p < .05$) and "treatment × trial" (distance $F_{3,108} = 3.37$, $p < .05$) in the wild-type group, where naltrexone significantly potentiated swimming latencies and distances in the second (Figures S4A-B) ($p < .001$, $p < .01$), third ($p < .05$) and fourth ($p < .05$) match trials, but not in the location trial. This result implies that naltrexone-treated wild-type mice solved the matching-to-location task better because of their superior short-term memory. In contrast, the working memory of the GluA1-S845A mutant mice was not affected by chronic naltrexone treatment.

The lack of phosphorylation at S845 seemed to impair the working memory of the control condition as revealed by the significant main effect of "genotype" (Figures S4A-B) ($F_{1,36} = 45.45$, $p < .001$; $F_{1,36} = 36.80$, $p < .001$) and significant post hoc differences in the first (latency $p < .001$; distance $p < .01$) and second (latency $p < .05$) trials. This was corroborated by the observation that the GluA1-S845A mutants had a lower swimming speed during the first sample trial (Figure S4E) ($p < .001$). Analyses of the percentages of time spent, and distances swum, in

the platform location quadrant from the previous day during the first sample trial showed that the saline-treated GluA1-S845A mutants more frequently visited the platform location from the previous day (Figures S4C-D) (distance $p < .05$).

Because chronic naltrexone treatment improves short-term memory in wild-type but not GluA1-S845A mutant mice (Figures S4A-B), we suggest a critical role of GluA1-S845 phosphorylation-dependent AMPAR trafficking on working memory. Although the GluA1-S845A mutants reached a similar proficiency level as the wild-type control at the end of the training, there was an apparent and significant deficit on the sample trial induced by the GluA1-S845A mutation, possibly due to a better retention of the platform location from the previous day in the mutants. Indeed, in the matching-to-sample protocol applied in our study, the repetition of a set of four consecutive trials/day with the hidden platform location and starting position unchanged between the trials would increase the probability of retention of the escape position, even though the platform location and starting position were modified daily. Since GluA1-S845A mutation does not affect learning in the cued version of the water and Barnes maze, the working memory deficits in the mutants is unlikely to result from non-mnemonic dysfunction.

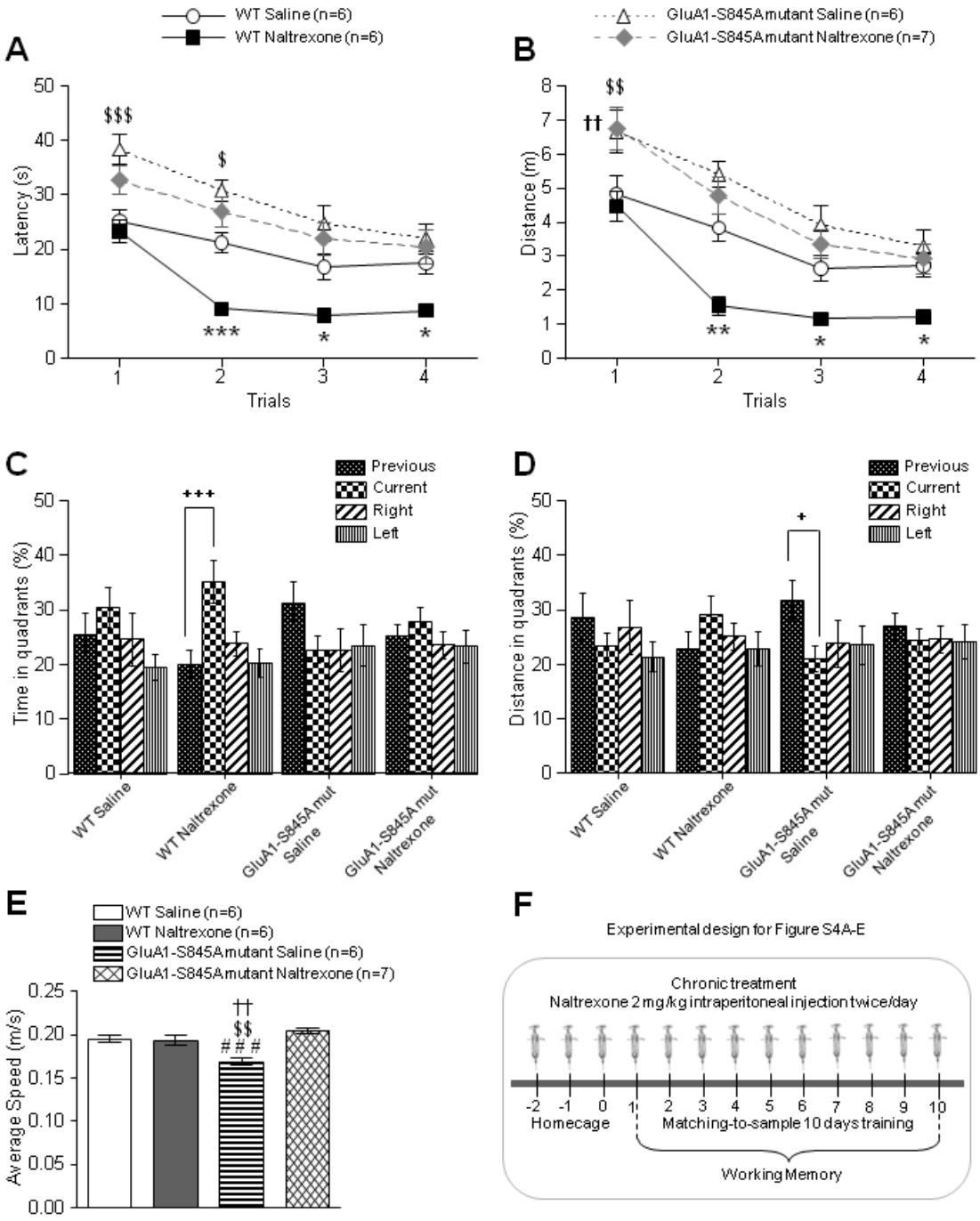

Figure S4. Effect of chronic naltrexone administration on working memory in GluA1-S845A mutants and wild-type littermates. Points and whiskers are means ± SEMs. Chronic intraperitoneal injection with naltrexone 2 mg/kg twice/day started 3 days (-2, -1, 0 on the timeline) before the first day of the training period of the task during which the mice were given 4 trials/day to locate the hidden platform for 10 days (1 to 10 on the timeline). The platform position was changed each day **(F)**. In the wild-type mice (saline-treated wild-type = open

circles; naltrexone-treated wild-type = filled squares) (n = 6/group), naltrexone treatment shortened the escape latency **(A)** and the distance swum **(B)** to the hidden platform in match trials compared to the sample trial. In contrast, among the GluA1-S845A mutants, the antagonist had no effect (saline-treated GluA1-S845A mutants = open triangles (n = 6); naltrexone-treated GluA1-S845A mutants = filled diamonds (n = 7)). However, saline- and naltrexone-treated GluA1-S845A mutants had longer latencies and distances in the sample trial compared to the wild-type mice. Analyses of the percentages of time spent **(C)** and distance swum **(D)** in the previous platform location, current platform location and right and left quadrants during the sample trials showed that the saline-treated GluA1-S845A mutants preferred the previous platform location. **(E)** The speed during the sample trial was averaged over 10 days. Among-group comparisons revealed a slower swimming speed in the saline-treated GluA1-S845A mutants (n = 6/group). Significant differences among groups were determined using three-way ANOVAs, followed by Newman-Keuls's post hoc comparisons. * $p < .05$, ** $p < .01$ and *** $p < .001$ significant differences between saline- and naltrexone-treated wild-type mice, $ $p < .05$, $$ $p < .01$ and $$$ $p < .001$ significant differences between saline-treated wild-type mice and saline-treated GluA1-S845A mutants; # # # $p < .001$ significant difference between naltrexone- and saline-treated GluA1-S845A mutants; †† $p < .01$ significant difference between saline-treated GluA1-S845A mutants and naltrexone-treated wild-type mice; + $p < .05$, +++ $p < .001$ significant differences between previous and current platform locations.

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