Increased SK2 Channel-Mediated Negative Feedback on *N*-methyl-D-aspartate Receptors Impairs Synaptic Plasticity Following Context-Dependent Sensitization to Morphine

Supplemental Information

Supplemental Methods and Materials

Unpaired and Saline Challenge Mice

In the context unpaired group, mice received 5 mg/kg morphine in the locomotor activity (LMA) chamber to measure baseline LMA. The remaining 8, 10 and 15 mg/kg doses were given in various contexts. One week later they received the 5 mg/kg challenge dose in the LMA chamber to measure sensitization. After measuring sensitization, mice were immediately sacrificed and hippocampus was removed for electrophysiological or subcellular fractionation experiments.

For the saline challenge control group, mice were randomly assigned into groups that received morphine or saline paired with a novel context (a 41.5 x 41.5 x 30 cm LMA chamber equipped with photobeams (AccuScan Instruments)). Sixty minutes before morphine administration mice received an intraperitoneal (IP) injection of saline in the LMA chamber to habituate. After which IP injections of saline or escalating doses of morphine (5, 8, 10 and 15 mg/kg) were given and LMA was recorded for 90 minutes. Doses were given every 12 hours. One week later they received a saline challenge in the LMA chamber. After measuring sensitization, mice were immediately sacrificed and hippocampus was removed for electrophysiological or subcellular fractionation experiments.

Subcellular Fractionation and Western Blotting

Hippocampi were homogenized in a 0.32 M sucrose containing 0.1 mM $CaCl_2$ solution containing protease and phosphatase inhibitors (Sigma-Aldrich). A sample of this fraction was obtained as the total homogenate fraction. The rest of the solution was combined with 2 M sucrose and 0.1 mM $CaCl_2$ obtain a 1.25 M sucrose concentration. The samples were then overlaid with 1 M sucrose and placed in an ultracentrifuge and centrifuged at 100,000 g for 3 hours. The synaptosomal fraction, collected at the 1.25

M/1 M interface, was then incubated in a 20 Mm Tris pH 6 buffer containing 1% Triton X-100 for 20 minutes at 4°C with gentle agitation to solubilize the synaptic junctions which were collected by centrifugation at 40,000 g for 30 minutes at 4°C. Postsynaptic density (PSD) fractions were obtained by incubating the synaptic junctions in 20 Mm Tris pH 8 buffer containing 1% Triton X-100 for 20 minutes at 4°C with gentle agitation and collected by centrifugation at 40,000 g for 30 minutes at 4°C. The pellet containing the PSD fraction was resuspended in 1% SDS and kept at -80°C until use.

Either total homogenate or PSD fractions were loaded equally (10 mg) and separated in 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking in 5% non-fat dry milk, membranes were incubated with antibodies to NR1 (1:500, Pierce), NR2A (1:1000, Millipore), NR2B (1:1000, NeuroMab), SK2 (1:500, Sigma-Aldrich, [1]) or PP2A (1:1000; Millipore) either overnight at 4°C or for one hour at room temperature. After which they were probed with either IRDye 680-anti-mouse or IRDye 800 –anti-rabbit 1:5000 followed by scanning with Odyssey (LiCOR, Lincoln, NE) or with anti-rabbit-HRP 1:5000 followed by Amersham ECL Plus detection. Intensity of signal for NR2A, NR2B, NR1, SK2, and B-actin was measured using ImageJ software and each protein was normalized to its respective β-actin which did not change across treatment groups. Each treatment group was normalized to its saline treated control to calculate relative protein expression as a percentage of saline treatment groups.

For each protein analyzed, the ratio of that protein (NR2A, NR2B, NR1, SK2 and PP2A) to its corresponding β-actin was normalized to the saline treated group; therefore comparisons can be made between either S+M vs M+M or S+S vs M+S groups for each protein analyzed.

PP2A Activity Kit

Whole hippocampus was homogenized in buffer (1 ml/25 mg) containing 20 mM imidazole HCl, 2 mM EGTA, 2 mM EDTA and 1 mM benzaminidine and protease inhibitors (Sigma-Aldrich) followed by centrifugation at 2000 g for 5 minutes at 4°C. Five hundred micrograms of protein were incubated with 4 mg of anti-PP2A (Millipore) and 30 ml of Protein A agarose (Millipore) in pNPPSer/Thr assay

buffer for 2 hours rocking at 4°C. After which the beads were collected by centrifugation (1000 g, 2 minutes) and washed three times with TBS and once with pNPPSer/Thr assay buffer. Beads were collected by brief centrifugation and incubated for 10 minutes at 30°C with phosphopeptide and pNPPSer/Thr assay buffer. Equal amounts of phosphate solution standards and samples were loaded onto a 96 well plate with malachite green phosphate detection solution. After 15 minutes the optical density of the OD samples were read at 650 nm and concentration of the unknown samples were calculated by comparison to the standard curve.

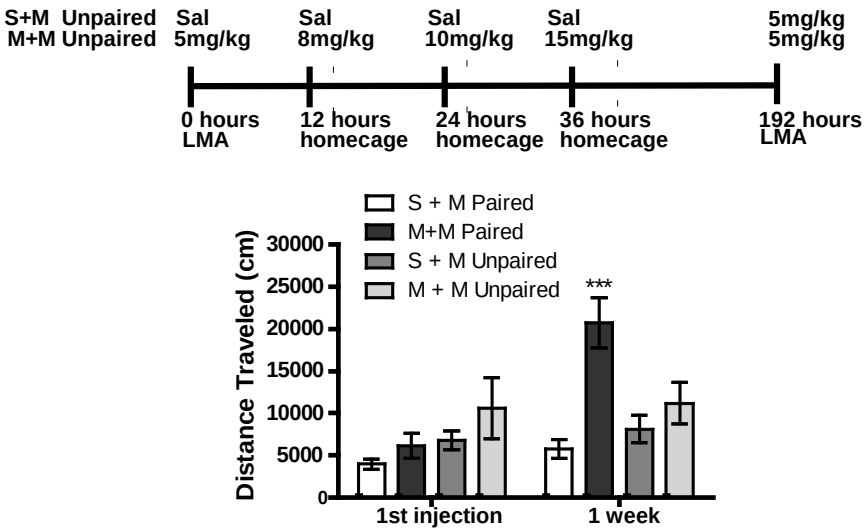

Figure S1. Mice that receive escalating doses of morphine unpaired with context do not display locomotor sensitization. Mice receive the first 5 mg/kg morphine in the LMA chamber in order to measure baseline LMA whereas the 8, 10, and 15 mg/kg doses are given in the home cage. One week later the mice are given the 5 mg/kg challenge dose of morphine in the LMA chamber to determine if they are sensitized. For the first injection of 5 mg/kg morphine there are no differences in distance traveled between the groups. One week later mice that receive morphine paired with the LMA context (n = 15-20/group, $p < 0.0001$, Bonferroni's post-hoc tests) develop locomotor sensitization while the mice that receive the same morphine doses unpaired with the LMA context do not (n = 9-10/group, 2-way ANOVA, paired vs unpaired $p < 0.0001$, 1st injection vs 1 week $p < 0.01$ and interaction $p < 0.001$). LMA, locomotor activity; M, morphine; S, saline; Sal, saline.

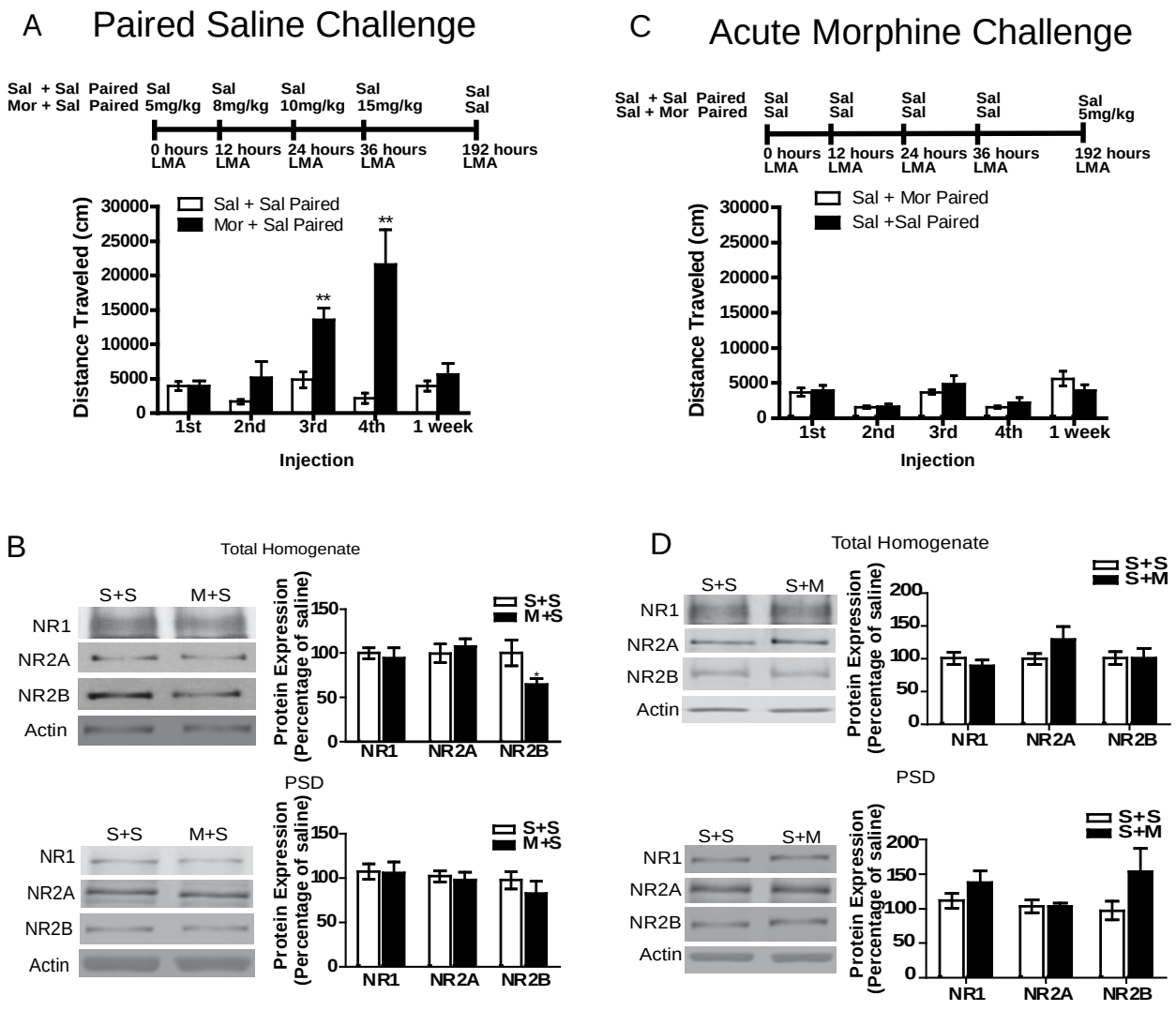

Figure S2. Saline challenge and acute morphine do not mediate changes in NMDAR subunit composition. (**A**) Mice have increases in LMA at the 10 and 15 mg/kg dose of morphine compared to saline mice (n = 6-7/group, $p < 0.01$, unpaired t-test). However, when they receive a saline challenge one week later there are no changes in LMA in either the S+S or M+S groups. (**B**) (Left panel) Representative western blots of NMDAR subunits in total homogenate and PSD fractions from hippocampus of paired S+S and M+S mice. (Right panel) Summary graphs representing the % of saline treatment as the mean ± SEM. In M+S mice compared to S+S, there is a significant decrease in NR2B in the total homogenate fraction (n = 8/group, $p < 0.05$) and no changes in the PSD fraction. (**C**) Paired S+S mice receive 4 injections of saline in the LMA chamber every 12 hours and one week later received a saline challenge. Paired S+M treated mice receive injections of saline every 12 hours in the context of the LMA chamber and one week later receive a 5 mg/kg morphine challenge. Mice that receive saline paired with the LMA chambers do not display changes in LMA even when challenged with a 5 mg/kg dose of morphine one week later. (**D**) Acute 5 mg/kg dose of morphine in the context of the LMA chamber does not affect expression of NMDAR subunits in total homogenate and PSD fractions (total homogenate n = 5-7/group; PSD n = 6/group). Data analyzed using unpaired t-tests. LMA, locomotor activity; NMDAR, *N*-methyl-D-aspartate receptors; M, morphine; Mor, morphine; PSD, postsynaptic density; S, saline; Sal, saline.

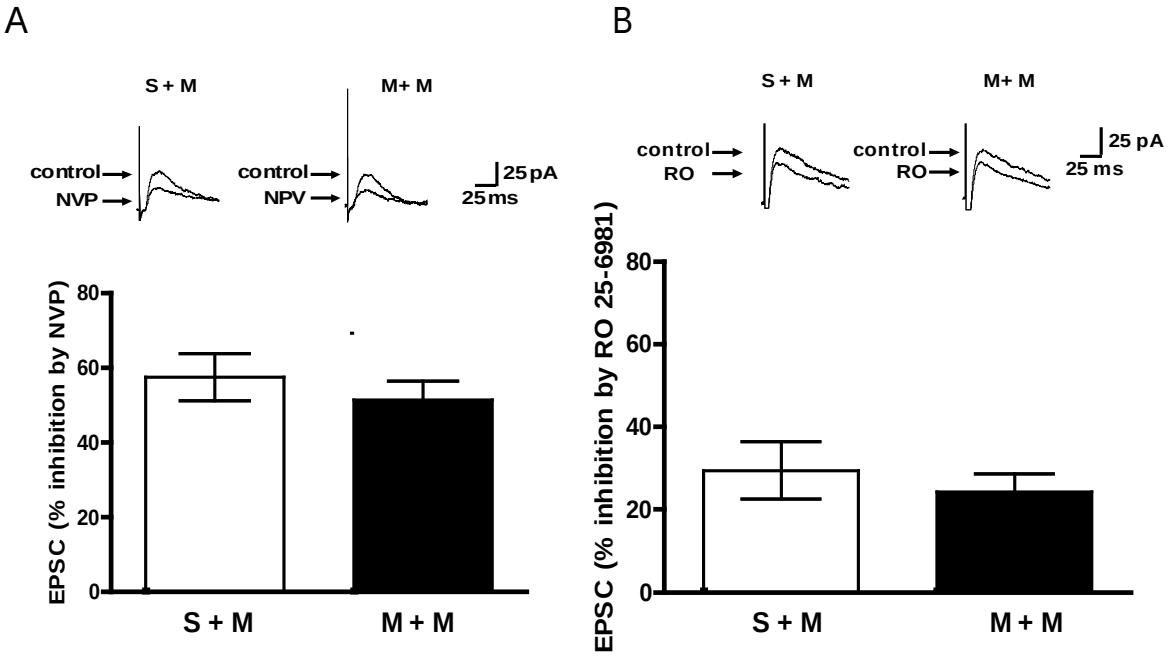

Figure S3. NMDAR currents in CA3 pyramidal neurons are not affected by context dependent sensitization to morphine. (**A**) (Upper panel) Representative trace of NMDAR EPSCs from CA3 pyramidal neurons before and after application of NVP. **(**Lower panel) Summary of % inhibition of EPSCs indicating that there is no difference in sensitivity to NVP between CA3 neurons from M+M and S+M mice (*n* = 4/group). (**B**) (Upper panel) Representative trace of NMDAR EPSCs from CA3 pyramidal neurons before and after application of RO 25-6921 in CA1 pyramidal neurons. (Lower panel) Summary of % inhibition of EPSCs indicating that there is no difference in sensitivity to RO between CA3 neurons from M+M and S+M mice (*n* = 4/group). EPSC, evoked excitatory postsynaptic currents; NMDAR, *N*-methyl-D-aspartate receptors; M, morphine; S, saline.

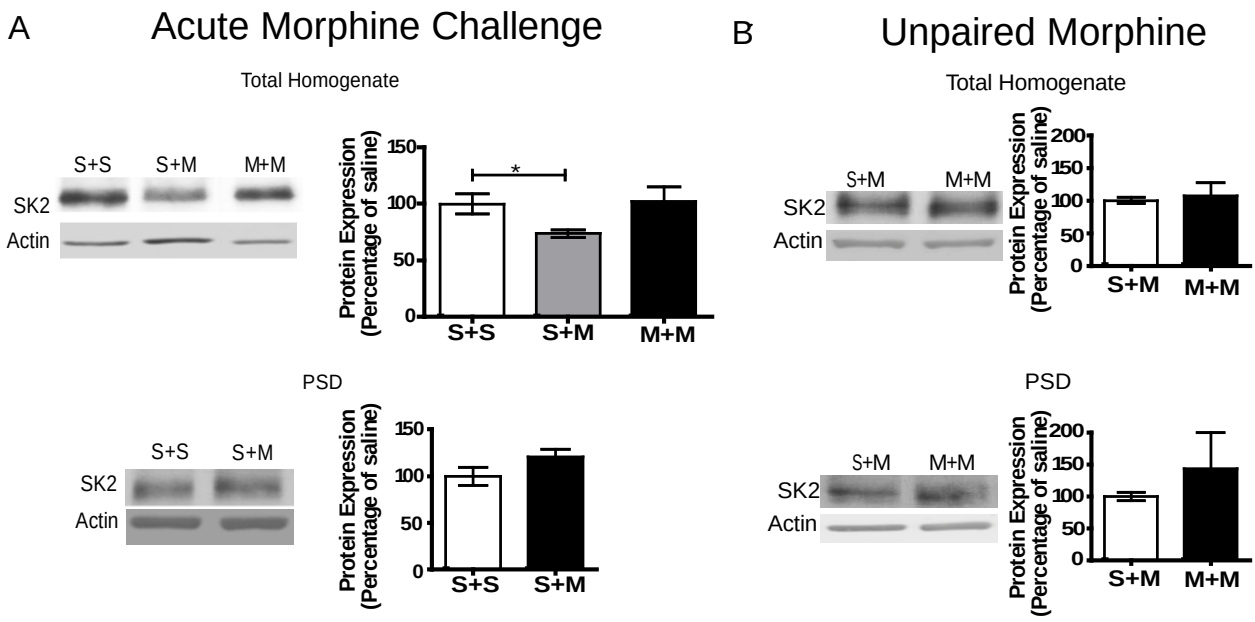

Figure S4. SK2 channel expression in the hippocampus is modulated by acute morphine and not by morphine administration unpaired with context. (**A**) Acute morphine decreases SK2 expression in the total homogenate (n = 6-7/group, $p < 0.05$) fraction and there are no differences in expression between paired S+S and M+M mice. There are no changes in SK2 expression in the PSD (n = 6/group). (**B**) Escalating doses of morphine given unpaired with context of the LMA chamber does not alter SK2 channel expression in either the total homogenate or PSD fractions (n = 4/group). Data analyzed using unpaired *t*-tests. LMA, locomotor activity; M, morphine; PSD, postsynaptic density; S, saline.

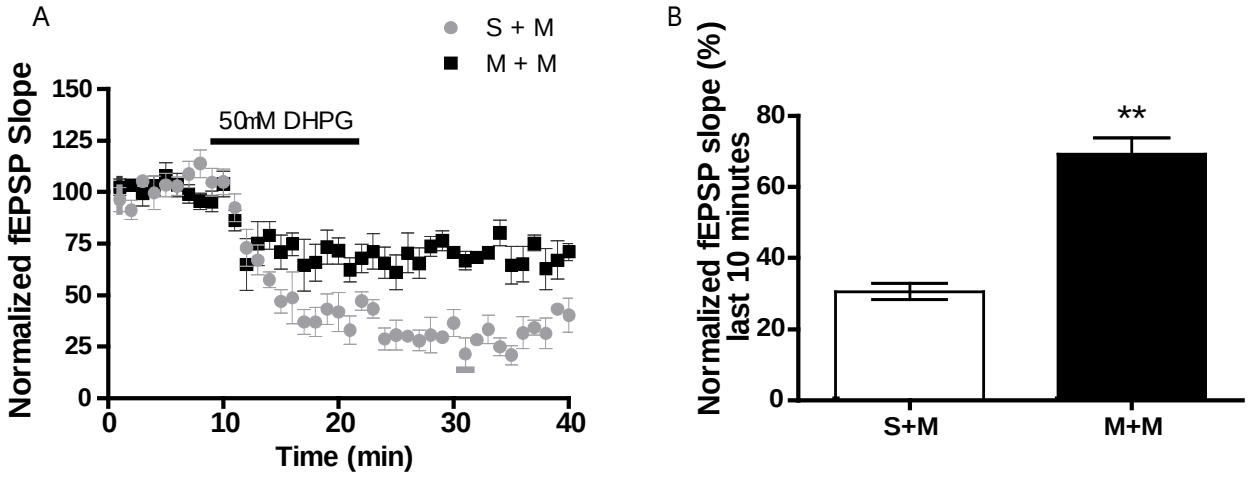

Figure S5. mGluR1/5 dependent long term depression in the hippocampus is affected by context dependent sensitization to morphine. (**A**) Time course of LTD induction by incubation with 50 mM DHPG for 15 minutes. (**B**) Comparison of magnitude of depression between S+M and M+M during the last ten minutes of recording indicating that there is greater depression in the S+M treated slices (n = 3) versus the M+M treated slices (n = 4). Data analyzed using unpaired *t*-test. $^{**}p < 0.01$. fEPSP, field excitatory postsynaptic potentials; LTD, long-term depression; M, morphine; mGluR, metabotropic glutamate receptor; PSD, postsynaptic density; S, saline.

Supplemental Reference

1. Hopf FW, Bowers MS, Chang SJ, Chen BT, Martin M, Seif T, *et al.* (2010): Reduced nucleus accumbens SK channel activity enhances alcohol seeking during abstinence. *Neuron*. 65:682-94.