

# Roles of NMDA NR2B Subtype Receptor in Prefrontal Long-Term Potentiation and Contextual Fear Memory

Ming-Gao Zhao,<sup>1,2,4</sup> Hiroki Toyoda,<sup>2,4</sup>  
Yong-Seok Lee,<sup>2,3,4</sup> Long-Jun Wu,<sup>1,2,4</sup>  
Shanelle W. Ko,<sup>2,4</sup> Xue-Han Zhang,<sup>1,4</sup> Yongheng Jia,<sup>2</sup>  
Fanny Shum,<sup>2</sup> Hui Xu,<sup>2</sup> Bao-Ming Li,<sup>1,\*</sup>  
Bong-Kiun Kaang,<sup>2,3</sup> and Min Zhuo<sup>1,2,\*</sup>

<sup>1</sup>Laboratory of Higher Brain Functions  
Institute of Neurobiology

Fudan University  
220 Han-Dan Road  
Shanghai 200433  
China

<sup>2</sup>Department of Physiology  
Faculty of Medicine  
University of Toronto  
1 King's College Circle  
Toronto, Ontario M5S 1A8  
Canada

<sup>3</sup>Department of Biological Sciences  
College of Natural Sciences  
Seoul National University  
Seoul 151-742  
Korea

## Summary

Cortical plasticity is thought to be important for the establishment, consolidation, and retrieval of permanent memory. Hippocampal long-term potentiation (LTP), a cellular mechanism of learning and memory, requires the activation of glutamate N-methyl-D-aspartate (NMDA) receptors. In particular, it has been suggested that NR2A-containing NMDA receptors are involved in LTP induction, whereas NR2B-containing receptors are involved in LTD induction in the hippocampus. However, LTP in the prefrontal cortex is less well characterized than in the hippocampus. Here we report that the activation of the NR2B and NR2A subunits of the NMDA receptor is critical for the induction of cingulate LTP, regardless of the induction protocol. Furthermore, pharmacological or genetic blockade of the NR2B subunit in the cingulate cortex impaired the formation of early contextual fear memory. Our results demonstrate that the NR2B subunit of the NMDA receptor in the prefrontal cortex is critically involved in both LTP and contextual memory.

## Introduction

Glutamate NMDA receptors (NMDARs) are required for the synaptic plasticity associated with the mechanisms of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). Native NMDARs are composed of NR1, NR2 (A, B, C, and D), and NR3 (A and B) subunits. The formation of functional NMDARs requires

a combination of NR1, an essential channel-forming subunit, and at least one NR2 subunit. It is known that the NR2A and NR2B subunits predominate in the forebrain, where they determine many of the functional properties of NMDARs (Loftis and Janowsky, 2003; Monyer et al., 1994). Moreover, NMDARs of various subunit compositions may occur during early development and in different brain areas (Arrigoni and Greene, 2004; Monyer et al., 1994; Munoz et al., 1999; Ritter et al., 2002; Sheng et al., 1994).

In central synapses, NMDAR activation is required for LTP induction (Bear and Kirkwood, 1993; Bliss and Collingridge, 1993; Lisman, 2003), and both NR2A- and NR2B-dependent signaling pathways are believed to be involved in hippocampal long-term synaptic plasticity in adult mice (Kiyama et al., 1998; Kohr et al., 2003; McHugh et al., 1996). For example, studies of transgenic mice overexpressing NMDAR NR2B in the adult forebrain and KIF17 transgenic mice with upregulated NR2B expression demonstrate the important contribution made by NR2B subunits to hippocampal LTP and behavioral learning (Tang et al., 1999; Wong et al., 2002). A recent study suggests that hippocampal LTP is mediated by NMDARs containing the NR2A but not NR2B subunit (Liu et al., 2004). This finding suggests that the NMDA NR2B receptors in the hippocampus may not contribute to learning-related synaptic potentiation; however, no behavioral study has demonstrated the inhibitory effect that NR2B antagonists may have on learning when injected locally into the hippocampus.

Forebrain structures, including the anterior cingulate cortex (ACC), are thought to be important for higher brain function, and neuronal activity in these areas plays important roles in emotion, learning, and memory (Devinsky et al., 1995; Wiltgen et al., 2004; Zhuo, 2002). Moreover, activity-dependent gene imaging and regional inactivation studies have shown that the ACC is involved in remote fear and spatial memory (Frankland et al., 2004; Maviel et al., 2004). Although CaMKII is suggested to be required for LTP and permanent memory in the ACC (Frankland et al., 2001), the synaptic mechanisms underlying LTP and memory in the prefrontal cortex have been far less investigated than in hippocampal synapses. In terms of the acquisition of fear memory, the majority of previous studies have been done in the amygdala (Rodrigues et al., 2004). Even though neurons in the prefrontal cortex have projections to the amygdala (Cassell and Wright, 1986) and the role of the prefrontal cortex in fear extinction has been reported (Milad and Quirk, 2002; Santini et al., 2004), only a few conflicting results are available on the contributions of the prefrontal and/or ACC on fear memory acquisition (Gao et al., 2004; Han et al., 2003; Johansen and Fields, 2004; Tang et al., 2005).

In the present study, we propose that the direction of NMDAR-dependent synaptic plasticity in the ACC is determined not only by the different subunits but also by the NR2B/NR2A subunit composition and receptor phosphorylation. Integrative approaches, including electrophysiological, biochemical, pharmacological, and be-

\*Correspondence: bml@fudan.edu.cn (B.-M.L.); min.zhuo@utoronto.ca (M.Z.)

<sup>4</sup>These authors contributed equally to this work.

havioral techniques, were used to show that the NMDA NR2A and NR2B receptors are critical for the induction of LTP in the ACC. Finally, using direct brain electroporation of siRNA or a pharmacological antagonist, we provide evidence that NR2B receptors in the ACC contribute to the formation of contextual fear memory.

## Results

### Cingulate LTP in Adult Mice Requires NMDA Receptor Activation

We performed whole-cell patch-clamp recordings in visually identified pyramidal neurons in layer II/III of ACC slices and identified pyramidal neurons by injecting depolarized currents into neurons to induce action potentials. The typical firing pattern of pyramidal neurons showed significant firing frequency adaptation (Tsvetkov et al., 2002), whereas interneurons showed fast-spiking action potentials followed by pronounced hyperpolarization. We also identified pyramidal neurons based on the pyramid shape of their somata by putting Lucifer yellow into the intracellular solution (Figure 1A). To determine whether synaptic transmission undergoes LTP, we paired synaptic stimulation with postsynaptic depolarization (also referred to as “pairing training”) (80 pulses of presynaptic stimulation at 2 Hz in layer V with postsynaptic depolarization at +30 mV) (Artola et al., 1990; Tsvetkov et al., 2004). The pairing training produced a significant, long-lasting potentiation of synaptic responses (mean = 160.0% ± 10.8% of baseline, n = 15; t test, p < 0.001 versus baseline responses before the pairing training; Figure 1B). In some neurons (n = 5), which were recorded over a long period, LTP persisted for at least 90 min (Figure 1C). In the control group, neurons were not subjected to pairing training, and synaptic responses were not significantly altered over the entire recording period (last 5 min mean = 98.1% ± 5.8% of first 5 min baseline response, n = 5; t test, p = 0.66; Figure 1D).

To determine whether NMDAR activation is required for cingulate LTP induction, we applied a selective NMDAR antagonist, AP-5 (50 μM), and found that LTP was completely blocked (97.8% ± 5.7%, n = 7; Figure 1E). Furthermore, LTP induction was completely abolished by 11 mM BAPTA in the pipette solution (106.1% ± 10.3%, n = 7; Figure 1F), indicating that cingulate LTP is dependent on the activation of NMDARs and elevated postsynaptic Ca<sup>2+</sup> concentrations.

### Cingulate LTP Expression Depends on a Postsynaptic Mechanism

Both presynaptic and postsynaptic mechanisms have been proposed to contribute to LTP expression (Nicoll and Malenka, 1995). For example, in the CA3 region, the presynaptic expression of LTP is accompanied by altered paired-pulse facilitation (PPF) (Zalutsky and Nicoll, 1990). To determine whether presynaptic mechanisms are involved in expression of LTP in the ACC, we measured PPF before and after LTP induction. As shown in Figure 2A, PPF was not altered after the LTP induction protocol (ratios of EPSC2/EPSC1 = 1.8 ± 0.2 and 1.6 ± 0.2, respectively, before and 25 min after LTP induction, n = 5; paired t test, p = 0.39), whereas synap-

tic responses were significantly enhanced in the same slices (EPSC1 = 149.0% ± 17.0% of baseline, n = 5; t test, p < 0.05 versus baseline; Figure 2B). To determine the possible role of presynaptic mechanisms in ACC LTP expression, we also tested whether NMDAR-mediated currents were altered after LTP induction. We predicted that NMDAR-mediated currents would be enhanced if presynaptic mechanisms were involved. However, after LTP induction, NMDAR-mediated currents were decreased to 76.8% ± 5.0% of baseline (n = 7; t test, p < 0.01; see Figure S1 in the Supplemental Data available online). This result suggests that the expression of LTP in the ACC depends on a postsynaptic mechanism.

### Requirement of NR2A and NR2B Subunits

Next, we examined the contribution of both NR2A and NR2B to the induction of cingulate LTP by application of the specific NR2A subunit antagonist NVP-AAM077 (IC50 of 14 nM and 1.8 μM for NR1/NR2A and NR1/NR2B, respectively) (Auberson et al., 2002) and the NR2B subunit antagonist Ro25-6981 (IC50 of 9 nM and 52 μM for NR1/NR2B and NR1/NR2A, respectively) (Fischer et al., 1997) or ifenprodil (IC50 of 0.34 μM and 146 μM for NR1/NR2B and NR1/NR2A, respectively) (Williams, 1993). As shown in Figure 3A, cingulate LTP was significantly reduced but not completely blocked by two different doses of NVP-AAM077 (0.1 μM: 134.9% ± 6.9%, n = 7; t test, p < 0.01; 0.4 μM: 131.6% ± 11.9%, n = 9; t test, p < 0.01). This partial blockade of LTP raises the possibility that the NR2B subunit contributes to the induction of cingulate LTP. We then tested the effects of NR2B subunit-selective antagonists on the induction of LTP. We found that LTP was partially reduced by two doses of Ro25-6981 (0.3 μM: 132.8% ± 7.4%, n = 9; p < 0.01; 3 μM: 126.8% ± 7.5%, n = 5; p < 0.05; Figure 3B) or 3 μM ifenprodil (128.1% ± 7.4%, n = 7; p < 0.05; Figure 3C). Because NR2A or NR2B antagonists only partially blocked LTP, we tested the effects of combinations of the two antagonists. LTP was completely blocked by the combination of 0.4 μM NVP-AAM077 and 0.3 μM Ro 25-6981 (102.7% ± 7.1%, n = 6; Figure 3D). These results provide evidence that both NR2B and NR2A NMDAR subunits contribute to the formation of cingulate LTP.

### Involvement of the NR2B Subunit Is Not Dependent on LTP Induction Protocols

To test whether the involvement of NR2A and NR2B subunits is dependent on the specific LTP induction paradigm used, we tested the role of NR2A and NR2B using two different induction protocols. First, we used a protocol (EPSPs-APs protocol, see Experimental Procedures) based on the coincidence of postsynaptic action potentials (APs) and unitary excitatory postsynaptic potentials (EPSPs, 10 ms ahead) to induce LTP (Bi and Poo, 1998; Markram et al., 1997) and found that this protocol produced a significant, long-lasting potentiation (141.9% ± 11.9%, n = 5; t test, p < 0.05 versus baseline; Figure 4A). Moreover, this potentiation was completely blocked by either 0.3 μM Ro25-6981 (114.4% ± 11.5%, n = 5) or 0.4 μM NVP-AAM077 (102.0% ± 11.5%, n = 5; Figures 4B and 4C). Second,

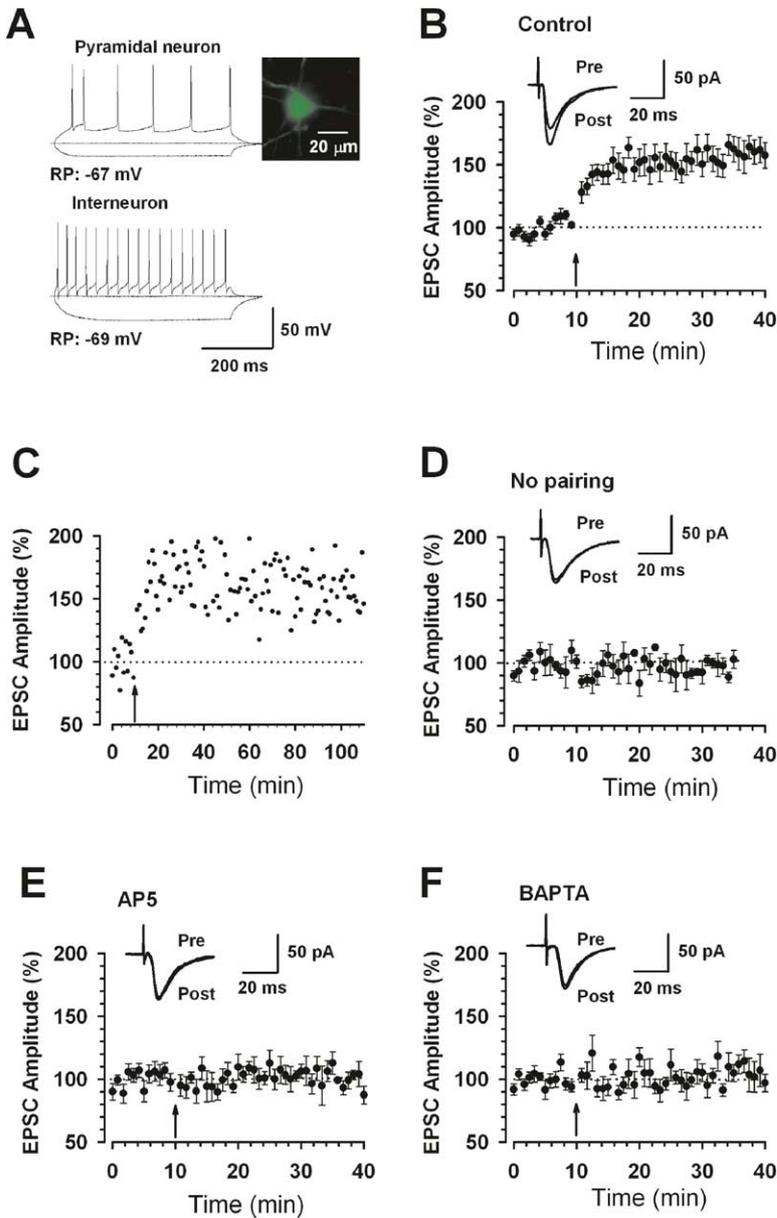


Figure 1. LTP Is Induced by Postsynaptic NMDA Receptor Activation in the ACC

(A) Current-clamp recordings to identify pyramidal neurons (upper) and interneurons (bottom) by current injections of  $-100$ ,  $0$ , and  $100$  pA. A labeled pyramidal-like neuron is shown on the top right. RP, resting membrane potential.

(B) LTP was induced in pyramidal neurons ( $n = 15$ ) in adult ACC by the pairing training protocol (indicated by an arrow). The insets show averages of six EPSCs 5 min before and 25 min after the pairing training (arrow). The dashed line indicates the mean basal synaptic responses.

(C) A example showing the long-lasting synaptic potentiation. Pairing training is indicated by an arrow.

(D) Basic synaptic transmission showing no change during recording without applying pairing training. The insets show averages of six EPSCs at the time points of 5 (pre) and 35 (post) min during the recording.

(E and F) LTP was completely blocked by bath application of AP-5 ( $n = 7$ ) or addition of BAPTA ( $n = 7$ ) in the intracellular solution. The insets show averages of six EPSCs 5 min before and 25 min after the pairing training (arrow). The dashed line indicates the mean basal synaptic responses.

we induced LTP using theta-burst stimulation (TBS) and found that TBS induced a significant LTP in the ACC ( $144.1\% \pm 11.8\%$ ;  $n = 5$ ;  $t$  test,  $p < 0.05$ ; Figure 4D). Similarly, LTP was blocked by  $0.3 \mu\text{M}$  Ro25-6981 ( $105.5\% \pm 8.3\%$ ,  $n = 7$ ) or  $0.4 \mu\text{M}$  NVP-AAM077 ( $97.0\% \pm 13.4\%$ ,  $n = 5$ ; Figures 4E and 4F). Taken together, these results indicate that the role of NR2B in the induction of LTP does not depend on the induction paradigm.

#### NR2A- and NR2B-Mediated EPSCs in the ACC and Hippocampus

It has been shown that the NMDA NR2B receptors are not responsible for LTP induction in the hippocampus (Liu et al., 2004). Then what are the mechanisms underlying the different roles of NR2B in cingulate and hippocampal LTP? One possible mechanism is that

NR2B-containing receptors may contribute more to total NMDA currents in ACC synapses than in hippocampal synapses. To test this, we used pharmacological antagonists for NR2A or NR2B and examined synaptically induced NMDAR-mediated EPSCs. Bath application of  $0.1 \mu\text{M}$  or  $0.4 \mu\text{M}$  NVP-AAM077 depressed total NMDAR-mediated currents by  $59.0\% \pm 4.3\%$  ( $n = 5$ ) and  $63.2\% \pm 1.7\%$  ( $n = 6$ ) in the ACC, respectively (Figure 5). On the other hand, application of  $0.3 \mu\text{M}$  or  $3 \mu\text{M}$  Ro25-6981 attenuated total NMDAR-mediated currents by  $18.0\% \pm 1.9\%$  ( $n = 5$ ) and  $18.1\% \pm 3.4\%$  ( $n = 7$ ) in the ACC, respectively, showing that  $0.3 \mu\text{M}$  Ro25-6981 was sufficient to block the NR2B-mediated currents (Figure 5). We repeated the experiment in hippocampal CA1 neurons and found that  $0.1 \mu\text{M}$  or  $0.4 \mu\text{M}$  NVP-AAM077 depressed total NMDAR-mediated currents by  $54.4\% \pm 3.2\%$  ( $n = 5$ ) and  $73.1\% \pm 1.7\%$  ( $n = 6$ ), respec-

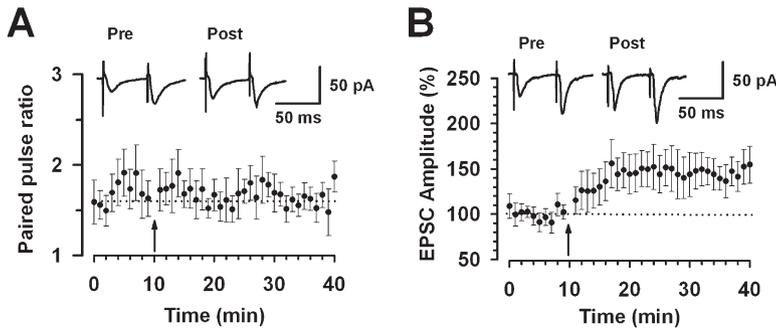


Figure 2. Paired-Pulse Facilitation Was Not Changed during LTP in the ACC

(A) Paired-pulse facilitation (PPF: the ratio of EPSC2/EPSC1) was recorded with a 50 ms interval throughout LTP recordings ( $n = 5$ ). LTP was induced by pairing training. The dashed line indicates the mean PPF ratio. (B) LTP (shown as EPSC1 amplitude) was induced in ACC neurons ( $n = 5$ ). The insets show averages of six EPSCs 5 min before and 25 min after the pairing procedure (arrow). The dashed line indicates the mean basal synaptic responses.

tively ( $p < 0.001$ , Figures 5B–5E). However, unlike in the ACC, application of  $0.3 \mu\text{M}$  Ro25-6981 alone potentiated, rather than blocked, total NMDAR-mediated currents by  $51.3\% \pm 16.4\%$  in the hippocampus (Figure 5). This result is consistent with a recent report concerning the adult rat hippocampus suggesting an inhibitory relationship between NR2B and NR2A subunit-containing NMDARs (Mallon et al., 2005).

The inconsistent effect of Ro25-6981 on NMDAR-mediated currents between the ACC and hippocampus raises the possibility for distinctive NMDAR properties or the differential expression of NR2B and NR2A proteins in these two areas. To address this hypothesis, we calculated the relative percentages of NR2A- and NR2B-mediated currents by applying specific antagonists: NVP-AAM077 ( $0.4 \mu\text{M}$ ) and Ro25-6981 ( $0.3 \mu\text{M}$ ).

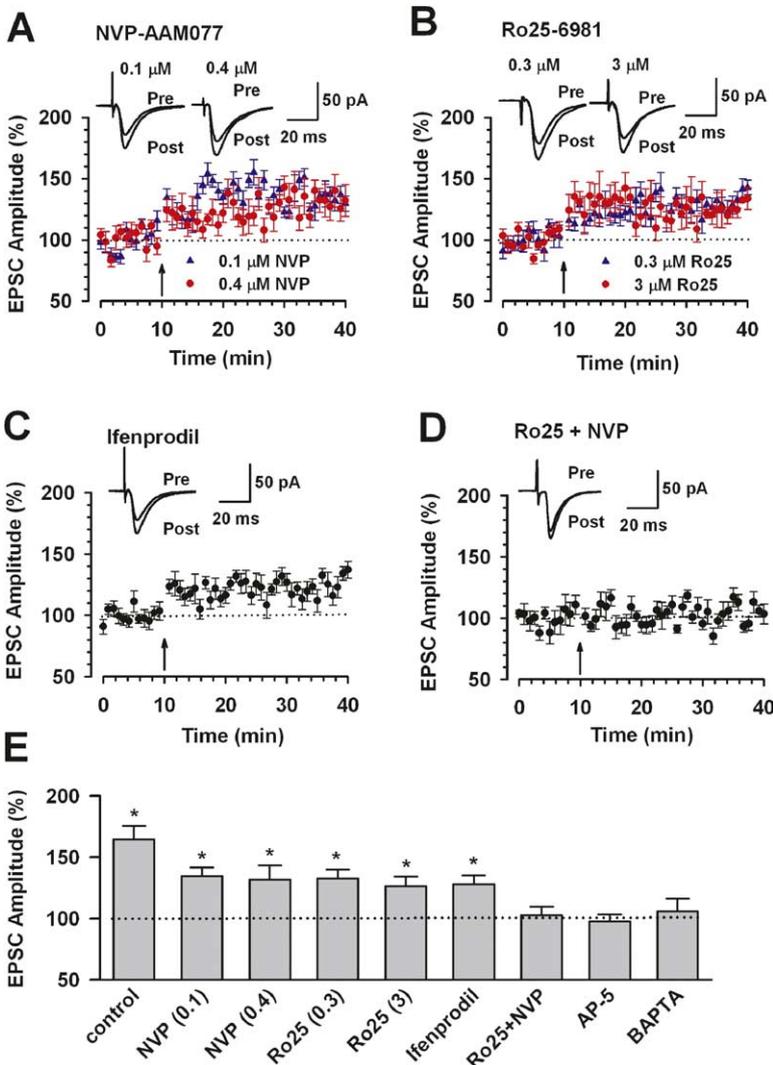
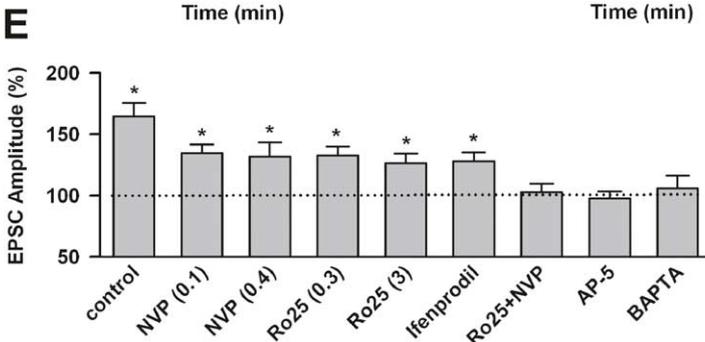


Figure 3. Contributions of the NR2A and NR2B Subunits to the Induction of LTP in the ACC

(A) LTP induced by the pairing training was partially depressed by  $0.1 \mu\text{M}$  ( $n = 7$ ) or  $0.4 \mu\text{M}$  ( $n = 9$ ) NVP-AAM077. (B) LTP was partially depressed by  $0.3 \mu\text{M}$  ( $n = 9$ ) or  $3 \mu\text{M}$  ( $n = 5$ ) Ro25-6981. (C) LTP was partially depressed by  $3 \mu\text{M}$  ifenprodil ( $n = 7$ ). (D) The coapplication of  $0.4 \mu\text{M}$  NVP-AAM077 and  $0.3 \mu\text{M}$  Ro25-6981 completely blocked LTP ( $n = 6$ ). (A–D) The insets show averages of six EPSCs 5 min before and 25 min after the pairing training (arrow). The dashed line indicates the basal synaptic responses. (E) Summary of the effects of NMDAR subunit antagonists or postsynaptic injection of BAPTA on LTP.  $*p < 0.05$  compared to baseline.



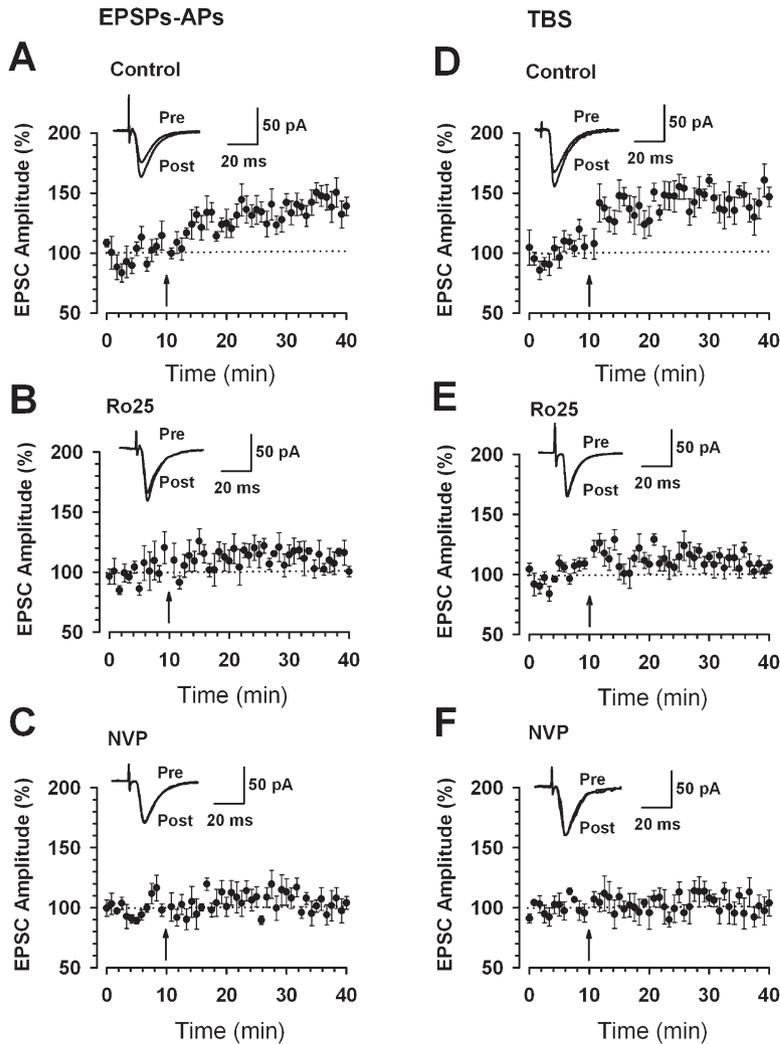


Figure 4. Effects of NR2A and NR2B Subunit Antagonists on LTP Induced by Two Other Protocols

(A–C) LTP was induced by a coincidence of postsynaptic action potentials and unitary EPSPs (10 ms ahead). (A) This protocol produced significant LTP in adult ACC neurons ( $n = 5$ ). (B) LTP was blocked by  $0.3 \mu\text{M}$  Ro25-6981 ( $n = 5$ ). (C) LTP was blocked by  $0.4 \mu\text{M}$  NVP-AAM077 ( $n = 5$ ).

(D–F) LTP was induced by the TBS protocol. (D) TBS induced significant LTP in the ACC of adult mice ( $n = 5$ ). (E) LTP was blocked by  $0.3 \mu\text{M}$  Ro25-6981 ( $n = 7$ ). (F) LTP was blocked by  $0.4 \mu\text{M}$  NVP-AAM077 ( $n = 5$ ).

(A–F) The insets show the average of six EPSCs 5 min before and 25 min after the EPSPs-APs protocol (arrow). The dashed lines indicate the basal synaptic responses.

The concentrations used were based on the results from Figure 5 and a recent study performed in the hippocampus (Liu et al., 2004). Application of NVP-AAM077 depressed total NMDAR-mediated currents by  $63.2\% \pm 1.7\%$ , and the addition of Ro25-6981 to the same neuron further reduced currents by  $13.7\% \pm 1.4\%$  ( $n = 6$ ) in the ACC (Figure 6). Reversing this order, by first applying Ro25-6981 and then adding NVP-AAM077, resulted in total NMDAR-mediated currents being depressed by  $18.0\% \pm 1.9\%$  and  $64.2\% \pm 3.0\%$  ( $n = 6$ ), respectively. Since Ro25-6981 and NVP-AAM077 show a similar effect on NMDAR-mediated currents regardless of the order of application, it is appropriate to use these antagonists to study NMDAR-mediated currents within the ACC; however, we cannot exclude the possibility that these antagonists also block triheteromers of NMDARs (NR1/2A/2B) (Hatton and Paoletti, 2005).

Next, we repeated the same experiment in the hippocampus. By first applying NVP-AAM077 ( $0.4 \mu\text{M}$ ) and then adding Ro25-6981 ( $0.3 \mu\text{M}$ ) to the same neuron, we found that the inhibitory effect of  $0.4 \mu\text{M}$  NVP-AAM077 in the hippocampus ( $73.1\% \pm 1.7\%$ ,  $n = 6$ ) was

significantly greater than that in the ACC neurons ( $63.2\% \pm 1.7\%$ ,  $n = 6$ ; *t* test,  $p < 0.01$ ; Figure 6B). The application of  $0.3 \mu\text{M}$  Ro25-6981 produced a further reduction in the hippocampus that was significantly smaller when compared to the inhibitory effect obtained in the ACC (ACC:  $13.7\% \pm 1.4\%$ ,  $n = 6$  versus hippocampus:  $6.6\% \pm 1.4\%$ ,  $n = 6$ , *t* test,  $p < 0.01$ ; Figure 6B). Kinetic analysis showed that the mean rise time and decay constants ( $\tau$ ) of NR2B-mediated NMDAR currents were greater than those of NR2A-mediated NMDAR currents, but these characteristics were similar in the ACC and hippocampus (Figures 6C and 6D).

#### Expression and Phosphorylation of the NR2B and NR2A Receptors

We next examined whether the different contributions made by NR2B and NR2A are due to the differential expression of NR2B and NR2A proteins in the ACC and hippocampus. We compared the relative subunit expression ratios of NR2B to NR2A in the ACC and hippocampus by Western blot analysis on total homogenates or synaptosomal membrane fractions. Western blot results showed that the basal expression level of NR2A

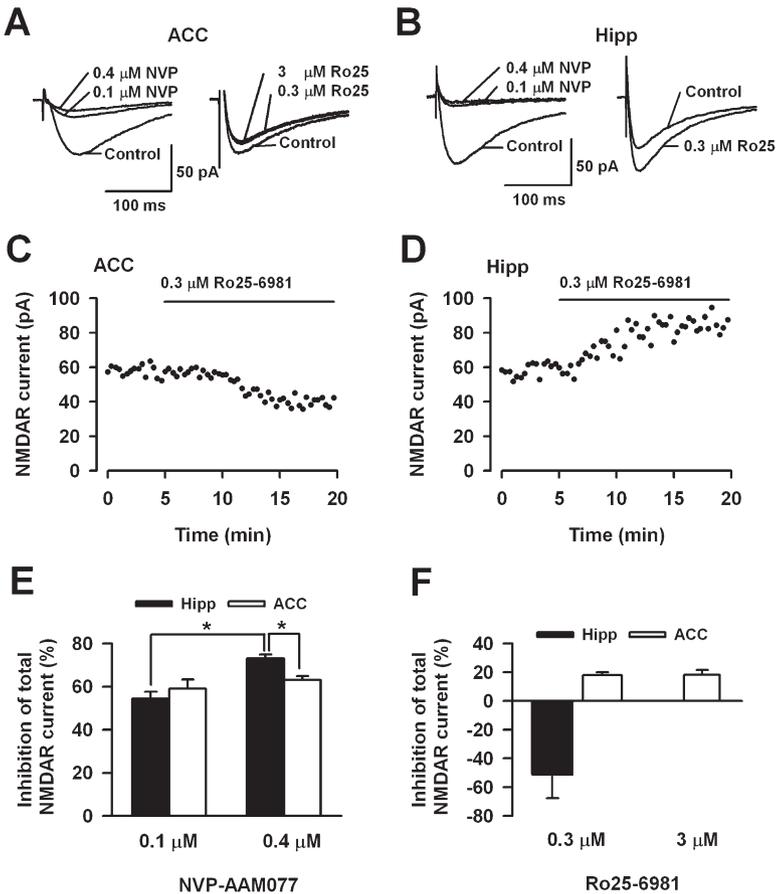


Figure 5. Effects of NR2A and NR2B Subunit Antagonists on NMDAR-Mediated EPSCs in the ACC and Hippocampus

(A) Representative traces show that bath application of different doses of NVP-AAM077 or Ro25-6981 depressed total NMDAR-mediated currents in the ACC.

(B) Representative traces show that bath application of different doses of NVP-AAM077 depressed total NMDAR-mediated currents in the hippocampus (left). In contrast, Ro25-6981 enhanced total NMDAR-mediated currents (right).

(C) Plot of peak EPSC amplitude versus time, showing that Ro25-6981 depressed total NMDAR-mediated current in the ACC.

(D) Plot of peak EPSC amplitude versus time, showing that Ro25-6981 enhanced total NMDAR-mediated current in the hippocampus.

(E) Summary of the effects of NVP-AAM077 on NMDAR-mediated currents in the ACC and hippocampus. \* $p < 0.05$ .

(F) Summary of the effects of Ro25-6981 on NMDAR-mediated currents in the ACC and hippocampus.

was consistently higher in the hippocampus compared to the ACC. In contrast, NR2B subunit expression was similar in both tissues (Figure 6E). In addition, the expression of PSD-95, a postsynaptic marker protein, was similar in the two tissues (Figure 6G). Thus, the NR2B/NR2A ratio in total homogenates was higher in the ACC than in the hippocampus ( $n = 7$ ;  $p < 0.05$ ; Figure 6F). Similarly, in synaptosomal membranes, the NR2B/NR2A ratio was also found to be higher in the ACC than in the hippocampus ( $n = 4$ ;  $p < 0.05$ ; Figure 6F). Thus, the higher contribution of NR2B in ACC synapses could explain the role of NR2B in LTP induction.

The function of NMDARs is regulated by its phosphorylation. Since tyrosine phosphorylation of NMDARs is thought to contribute to LTP (Collingridge and Singer, 1990; Lu et al., 1998), we examined the tyrosine phosphorylation levels of NR2A and NR2B subunits with anti-phosphotyrosine antibody to determine whether NMDARs are phosphorylated to different extents in the ACC and hippocampus (Figure 6G). NR2B subunits are found both intra- and extrasynaptically where they are involved in induction of LTD (Massey et al., 2004); however, because the current study focuses on the role of NR2A and NR2B subunits in LTP, we examined phosphorylation levels in synaptosomal membrane fractions. Tyrosine phosphorylation levels of both NR2A and NR2B in the ACC ( $n = 4$ ) were found to be significantly lower than in the hippocampus ( $n = 4$ ;  $p < 0.01$ ; Figure 6H).

### Reduction of NR2B Expression by siRNA Electroporation

Because LTP is believed to be an underlying mechanism in many forms of synaptic plasticity and memory, including fear conditioning mediated by the amygdala (Rodrigues et al., 2004; Rogan et al., 1997) and because our results demonstrate that the NR2B subunit of NMDA receptors is required for ACC LTP induction, we investigated the role of cortical NR2B in contextual fear conditioning (Rodrigues et al., 2004). To inhibit NR2B expression specifically in the prefrontal cortex, including the ACC, we bilaterally delivered siRNA against NR2B with a reporter plasmid encoding EGFP by microelectroporation (Wei et al., 2003) (Figure 8A). Two different experimental approaches were used to confirm the effectiveness and specificity of the NR2B siRNA. First, we performed Western blot analysis for NR2B and other membrane proteins. The expression levels of other NMDAR subunits (NR2A and NR1) and AMPA receptor subunit GluR1 were measured, and actin was used as a control in the ACCs of NR2B siRNA and control siRNA electroporated mice. We found that only NR2B protein was significantly reduced 4 days after electroporation ( $64.8\% \pm 4.6\%$  of NR2B expression compared to control siRNA electroporated control;  $n = 5$  mice,  $p < 0.01$ ; Figure 7A). The expression levels of NR2A, NR1, GluR1, and actin were not significantly changed.

NMDAR-mediated EPSCs were also recorded in neu-

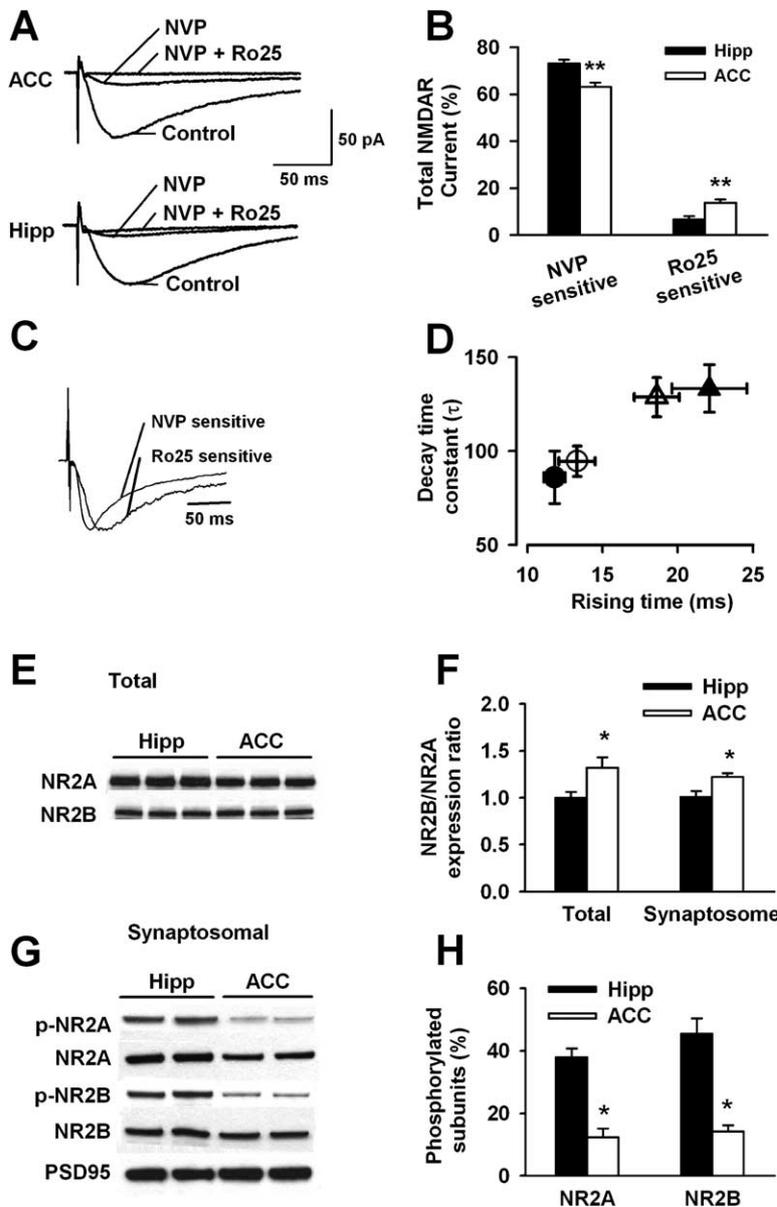


Figure 6. Comparison of NR2B- and NR2A-Mediated EPSCs and Their Expression Ratios in the ACC and Hippocampus

(A) Representative traces in control solution, in NVP-AAM077, and a combination of NVP-AAM077 and Ro25-6981 in ACC and hippocampal neurons.

(B) Percentage contribution by NVP-AAM077 (solid bars,  $n = 6$ ) and Ro25-6981 (open bars,  $n = 6$ ) sensitive EPSCs in the ACC and hippocampus.

(C) Ro25-6981-sensitive EPSC scaled to the peak of NVP-AAM077-sensitive EPSC.

(D) Time constant of EPSC decay ( $\tau$ ) versus the rising time (10%–90%) for EPSCs mediated by NR2A (circle) and NR2B (triangle) in the ACC (open) and hippocampus (solid).

(E) Representative Western blots of NMDAR subunit expression in total homogenates and (G) synaptosomal membrane fractions and of tyrosine-phosphorylated subunits of the ACC and hippocampus.

(F) Summary of the relative expression ratios of NR2B to NR2A in total homogenates and synaptosomal membrane fractions.

(H) Percentage of tyrosine-phosphorylated NR2A and NR2B subunits in the ACC and hippocampus ( $n = 7$ ). \* $p < 0.05$ , \*\* $p < 0.01$  versus the hippocampus.

rons from electroporated ACC slices. Consistently, we found that NR2B-mediated NMDA EPSCs were significantly reduced in NR2B siRNA-treated neurons ( $7.3\% \pm 0.7\%$ ,  $n = 11$  neurons from 5 mice versus control slices,  $12.6\% \pm 2.6\%$  of total NMDAR-mediated current,  $n = 5$  neurons from two mice;  $p < 0.05$ ; Figures 7B). Taken together, our data suggest that the effect of NR2B siRNA electroporation is target specific in terms of both protein expression and receptor function.

#### Reduced LTP by NR2B siRNA Electroporation

Having confirmed that NR2B expression was reduced in NR2B siRNA-treated neurons, we examined whether NR2B siRNA impairs the induction of LTP in ACC slices by using different induction protocols. First, we used a pairing training protocol to induce LTP and found that potentiation was significantly reduced in NR2B siRNA-

treated neurons as compared to neurons in control siRNA electroporated mice ( $n = 6$  for each group; Figures 7C and 7D). Second, TBS-induced potentiation was also significantly reduced ( $n = 6$ ,  $p < 0.05$ ; Figure 7). A similar reduction was found in LTP induced by EPSPs-APs protocol ( $n = 6$ ,  $p < 0.05$ ; Figure 7). LTP, induced by three different protocols, did not differ in mice injected with control siRNA compared to mice that did not receive an injection ( $p > 0.05$ ).

#### Genetic and Pharmacological Inhibition of Cortical NR2B Impairs Contextual Fear Memory Formation

Next, we examined whether NR2B subunit inhibition in adult mice impairs the formation of contextual fear memory. We trained mice 3 days after bilateral siRNA electroporation and then tested contextual fear memory by assessing freezing behavior in the same environ-

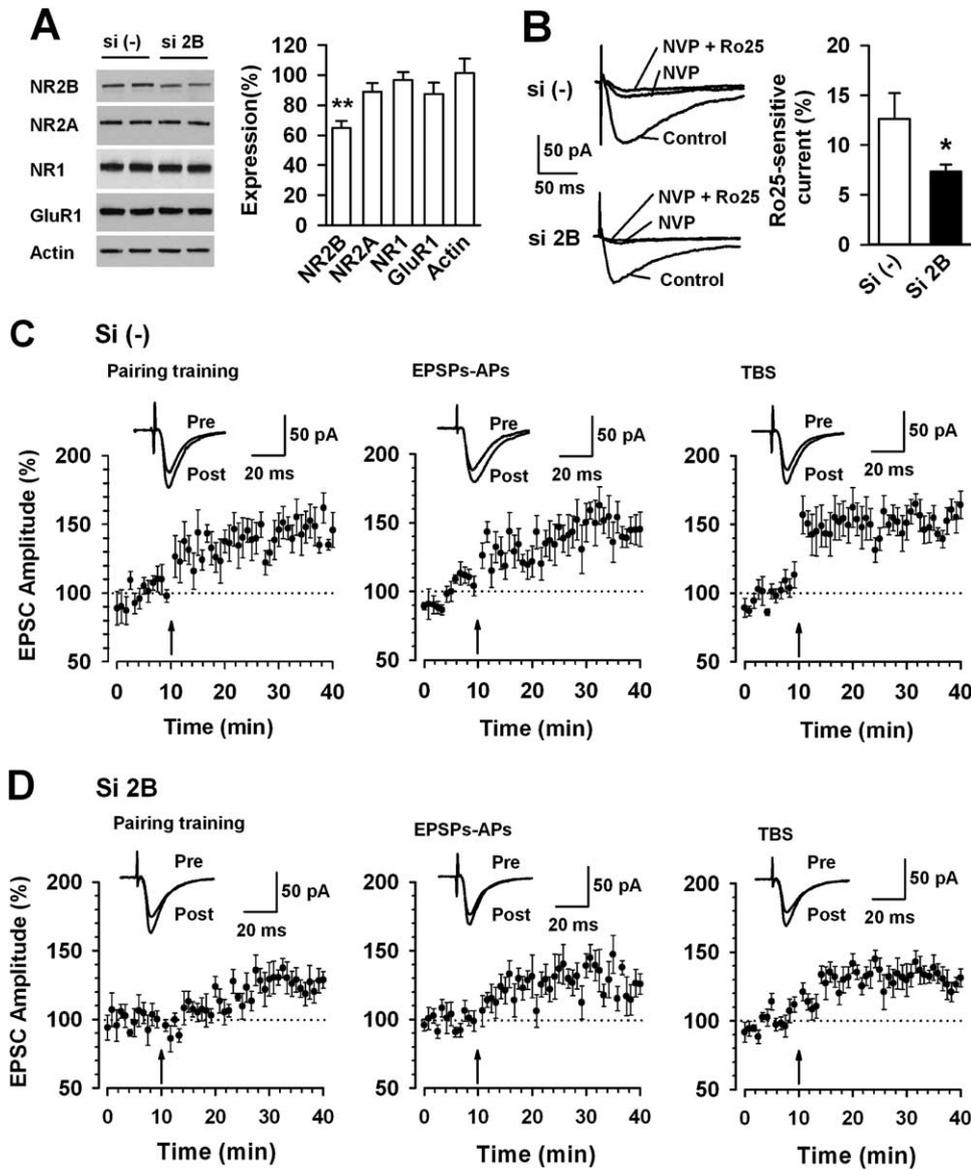


Figure 7. Electroporation of siRNA and Its Effect on NR2B Expression and LTP

(A) The effectiveness and specificity of NR2B siRNA electroporation. Western blots (left) were performed in tissue from siRNA-electroporated mice. Cortical tissue between the positive electroporation electrode and the injection site were dissected. Si (-), control siRNA; Si 2B, NR2B siRNA. Right column shows a summary of the Western blot analysis. Data are presented as a percentage of control siRNA treated tissues.  $n = 5$ ,  $**p < 0.01$ .

(B) Traces showing sample NR2A- and NR2B-mediated NMDAR currents from control siRNA (left upper) and NR2B siRNA (left down) electroporated ACC neurons. To calculate NR2B-mediated EPSCs, ACC neurons were sequentially treated with NVP-AAM077 and a combination of NVP-AAM077 and Ro25-6981. Statistical results (right) showing the Ro25-6981-sensitive component in adult ACC neurons from NR2B siRNA ( $n = 11$ ) and control siRNA ( $n = 5$ ) electroporated mice.  $*p < 0.05$ .

(C) LTP was induced by three different induction protocols in control siRNA electroporated neurons.

(D) Smaller LTP induced in NR2B siRNA electroporated neurons when compared with control siRNA.

(C and D) The insets show averages of six EPSCs 5 min before and 25 min after the pairing training (arrow). The dashed lines indicate the basal synaptic responses.

mental context after 24 hr. There was no difference in baseline freezing or in freezing immediately following the shock/tone pairing between groups; however, there was a significant difference between groups when tested in the contextual environment 1 day later ( $n = 8$  for EGFP only;  $n = 10$  for control siRNA;  $n = 8$  for shock only;  $n = 11$  for NR2B siRNA; one-way ANOVA,  $p =$

0.005; Figure 8C). NR2B siRNA-injected mice displayed significantly less freezing compared to GFP ( $p < 0.05$ ), shock only ( $p < 0.01$ ), and the control siRNA group ( $p < 0.05$ ), which suggests that NR2B in the cortex is involved in contextual fear memory processing. However, auditory fear memory was similar between all treatment groups (one-way ANOVA,  $p = 0.43$ ). Since the activation

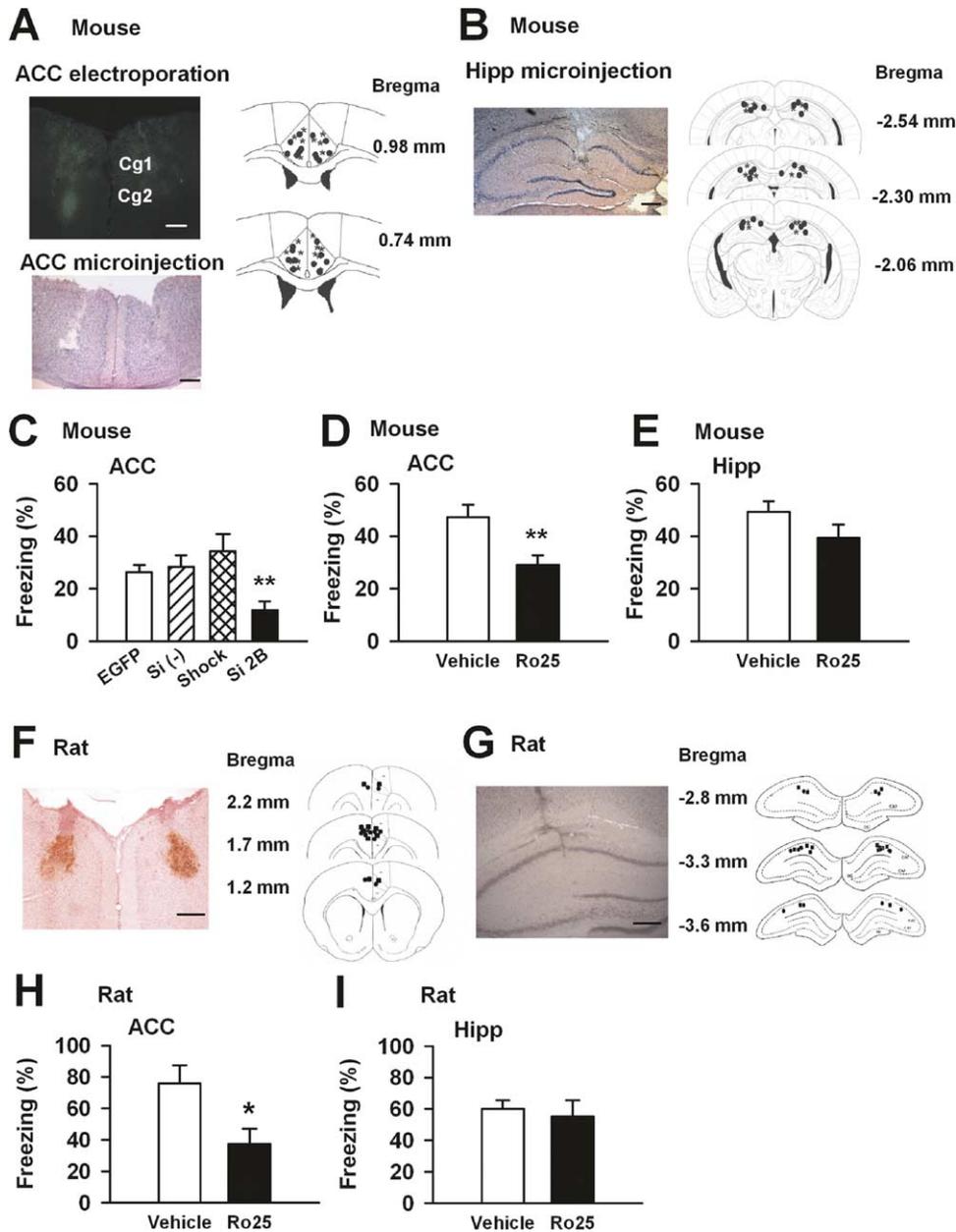


Figure 8. Effect of NR2B Blockade on Contextual Fear Memory

(A–E) Experiments performed in mice.

(A) ACC section (left upper) showing bilateral GFP-expressing neurons 4 days after electroporation. Cg1, anterior cingulate cortex area 1; Cg2, anterior cingulate cortex area 2. Representative coronal section (left down) showing ACC injection sites. Right column showing cannula tip placements in mice injected with Ro25-6981 (circles) or vehicle (asterisks) in the ACC. Scale bar, 300  $\mu$ m.

(B) Representative coronal section (left) showing hippocampal injection sites. Only one of the bilaterally injected sides is shown. Right column showing cannula tip placements in mice injected with Ro25-6981 (circles) or vehicle (asterisks) in the hippocampus. Scale bar, 300  $\mu$ m.

(C) The introduction of siRNA against NR2B into the adult cortex impaired contextual fear memory (open bars: EGFP only,  $n = 8$ ; hatched bar: EGFP + control siRNA,  $n = 10$ ; crossed bar: electric shock only,  $n = 8$ ; filled bar: NR2B siRNA<sup>+</sup> EGFP,  $n = 11$ ; t test,  $**p < 0.01$ ).

(D) Pharmacological blockade of NR2B in the cortex decreased contextual fear memory. Open bar: vehicle treated,  $n = 10$ ; solid bar: Ro25-6981 injected,  $n = 11$ ;  $*p < 0.05$ .

(E) Inhibition of NR2B in the hippocampus did not impair contextual fear memory. Open bar: vehicle treated,  $n = 9$ ; solid bar: Ro25-6981 treated,  $n = 12$ .

(F–I) Experiments performed in rats.

(F) Representative coronal section showing ACC injection sites. Scale bar, 600  $\mu$ m.

(G) Placement of cannulas in the rat hippocampus. Solid squares, vehicle; solid circles, Ro25-6981. Scale bar, 600  $\mu$ m.

(H) Inhibition of NR2B in the ACC impaired contextual fear memory. Open bar: vehicle treated,  $n = 6$ ; solid bar: Ro25-6981 treated,  $n = 6$ .  $*p < 0.05$ .

(I) Inhibition of NR2B in the hippocampus did not impair contextual fear memory. Open bar: vehicle treated,  $n = 7$ ; solid bar: Ro25-6981 treated,  $n = 6$ .

of NR2B was suppressed throughout the training and memory test, we cannot distinguish between the roles of NR2B during the acquisition and retrieval of fear memory. Recently, electric stimulation of the ACC was reported to induce fear memory 1 and 3 days after stimulation (Tang et al., 2005), which implies that the ACC may be involved in the acquisition of fear memory. To directly address this issue, we examined the effect of NR2B blockade using Ro25-6981. Microinjection of Ro25-6981 (2  $\mu$ g in 0.5  $\mu$ l per side) into the bilateral ACC before conditioning produced a significant reduction in freezing in the contextual environment (Ro25-6981,  $n = 11$ ; vehicle,  $n = 10$ ,  $p < 0.01$ ; Figure 8D). There was no difference in auditory fear memory between Ro25-6981- and vehicle-injected mice ( $p = 0.65$ ). In order to rule out any locomotor side effects, we tested locomotor activity in the open field after bilateral ACC microinjection of Ro25-6981 ( $n = 4$ ) or vehicle ( $n = 4$ ). There was no difference in locomotor activity between groups when recorded 15 min or 1 day after injection. Taken together, this suggests that the activation of NR2B in the ACC is important for the acquisition of contextual fear memory.

The hippocampus plays a role in contextual memory, and intrahippocampal injections of the NMDAR antagonist MK-801, which blocks NMDAR-mediated signaling, resulted in reduced contextual freezing (Bast et al., 2003; Fanselow, 2000; Fanselow et al., 1994). However, the effects of local hippocampal injections of NR2B antagonists on fear conditioning have not been examined. We infused Ro25-6981 (5  $\mu$ g in 0.5  $\mu$ l) bilaterally into dorsal hippocampi (Figure 8B) before conditional training. As in the ACC, hippocampal microinjection did not significantly affect baseline or immediate freezing behavior. Selective blockade of the NR2B subunit did not affect contextual or auditory fear memory when tested 1 day after training (contextual: Ro25-6981,  $n = 12$ ; vehicle,  $n = 9$ ;  $p = 0.159$ ; Figure 8E; auditory:  $p = 0.08$ ). We also microinjected the NR2B-specific antagonist into the ACC and hippocampus of rats and measured contextual and auditory fear memory. Similar to the results from mice, microinjection of Ro25-6981 (2  $\mu$ g in 1  $\mu$ l per side) bilaterally into the ACC (Figure 8F) before conditioning, produced a significant reduction in contextual fear memory ( $n = 6$  for each group,  $p < 0.05$ ; Figure 8H) when tested 1 day after training. However, intrahippocampal infusion of Ro25-6981 (0.1  $\mu$ g in 1  $\mu$ l) bilaterally into dorsal hippocampus (Figure 8G) before fear conditioning did not produce a significant impairment in the expression of contextual memory (Ro25-6981,  $n = 6$ ; vehicle,  $n = 7$ ;  $p = 0.67$ ; Figure 8I). Consistent with results in mice, Ro25-6981 did not affect auditory fear memory when injected in either the ACC or hippocampus (ACC: Ro25-6981,  $n = 6$ ; vehicle,  $n = 6$ ;  $p = 0.65$ ; hippocampus: Ro25-6981,  $n = 6$ ; vehicle,  $n = 7$ ;  $p = 0.78$ ; data not shown). Next, we wanted to test whether injections of a higher dose of Ro25-6981 (5  $\mu$ g in 1  $\mu$ l per side) bilaterally or multiple injections of the same dose (two per side) would affect either contextual or auditory fear memory. Neither single nor multiple injections of Ro25-6981 produced a significant impairment in the expression of contextual or auditory fear memory (Figure S2).

## Discussion

NMDA receptors play central roles in synaptic plasticity and memory in many brain regions, including the hippocampus (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). As in other regions in the central nervous system, glutamate is the major excitatory neurotransmitter in the ACC (Sah and Nicoll, 1991; Wei et al., 1999; Wu et al., 2005). However, the mechanism of LTP in the adult ACC has not been investigated. In the present study, we characterized postsynaptic, NMDAR-dependent cortical LTP and examined the roles of NMDAR subunits in LTP induction. Our data indicate that both NMDAR NR2A and NR2B subunits are involved in the formation of LTP in the ACC, a central region important for cognitive function. Furthermore, we provide evidence that NR2B in this area is involved in the formation of contextual fear memory.

### Molecular Mechanisms of Cortical LTP

In the present study, we characterized the mechanism of LTP in the prefrontal cortex and found that the induction of LTP depends on postsynaptic calcium concentration and NMDAR activation. As we could not detect any change in PPF after LTP induction, it is unlikely that the expression of LTP in the ACC depends on a presynaptic mechanism. Decreased NMDA currents after LTP induction (see Figure S1) are unlikely to support the hypothesis that LTP is due to the recruitment of silent presynaptic terminals, although more studies are needed to understand the mechanism for the depression of NMDA receptor-mediated responses. We also found that three different LTP induction paradigms that were developed for LTP induction in other brain regions can induce significant LTP in the adult ACC. Our results provide an opportunity to study the detailed physiological and/or pathological mechanisms underlying cortical plasticity.

In the present study, we found that the blockade of NR2A-containing NMDARs reduced the total NMDAR current by  $\sim 70\%$ , while the blockade of NR2B-containing NMDARs reduced NMDAR current by  $\sim 15\%$  in the ACC. However, both were effective in the attenuation of LTP, which is different from other studies that show a preferential role for NR2A in the hippocampus and perirhinal cortex (Liu et al., 2004; Massey et al., 2004). At least three possible mechanisms may explain this difference. First, unlike NR2A receptors, NR2B receptors are also found in extrasynaptic sites. Second, NR2B currents take longer to decay compared to NR2A currents. The third explanation involves the different tyrosine phosphorylation states of NR2A and NR2B subunits between the hippocampus and ACC. It has been suggested that NMDAR currents are regulated by non-receptor tyrosine kinases and phosphatases, such as Src and STEP, and that tyrosine phosphorylation of NR2A and NR2B is necessary to maintain and regulate NMDA currents (Kalia et al., 2004; Wang and Salter, 1994), which are necessary for induction of LTP at many synapses (Collingridge and Singer, 1990; Lu et al., 1998). Here, we found that the tyrosine phosphorylation states of NR2A and NR2B are lower in the ACC than in the hippocampus. We speculate that the highly phosphorylated state of both NMDAR subunits enables each

subunit to play a distinct role in determining the polarity of plasticity (LTP and LTD) in hippocampus. However, the roles of NMDAR subunits are not well differentiated in the ACC, which could be due to a lack of basal subunit phosphorylation. We suggest that these quantitative and qualitative changes in receptor subtypes may affect NMDA channel gating, synaptic localization, or/and coupling to signal transduction pathways that lead to LTP and LTD, even though NR2A and NR2B subunit-mediated EPSC kinetics showed no difference in the ACC and hippocampus. Furthermore, these changes in NMDARs in the adult ACC provide additional support for the role of NR2B receptors in the establishment, consolidation, and retrieval of permanent memory (Frankland et al., 2004; Hayashi et al., 2004).

### Contribution of NR2B in the Cortex to the Formation of Fear Memory

Recently, a role for the ACC in remote contextual fear memory (Frankland et al., 2004) and remote spatial memory (Maviel et al., 2004) has been reported. Cui et al. showed that NMDAR is needed for the long-term retention of fear memory after training (6–8 months) in inducible and reversible NR1 forebrain knockout mice (Cui et al., 2004). These studies emphasize the importance of the cortex as a permanent storage location for consolidated memory. However, another line of evidence suggests that the prefrontal cortex may play a critical role in the acquisition of several forms of memory, including fear memory in humans and rodents (Johansen and Fields, 2004; Knight et al., 2004; Morrow et al., 1999; Shallice et al., 1994). Nevertheless, the cortical synaptic mechanisms that mediate fear conditioning remain unclear. Here, we show that the blockade of NR2B using siRNA in the cortex impairs the early memory of contextual fear. By using an *in vivo* electroporation technique, we were able to specifically repress the expression and function of NR2B in living animals. However, due to the lack of a temporally specific gene silencing technique, we could not discern whether NR2B is involved in the acquisition or in the retrieval of fear memory. Because cortical activation was observed during the memory acquisition phase (Shallice et al., 1994) and electrical stimulation of the ACC was shown to induce fear memory (Tang et al., 2005), it is likely that NR2B in the cortex is involved in the acquisition of fear memory. NMDARs in both amygdala (Rodrigues et al., 2001) and hippocampus (Bast et al., 2003; Fanselow et al., 1994) have been reported to be involved in the formation of fear conditioning to context. We further examined this possibility using a pharmacological approach, which enabled us to dissect the temporal engagement of NR2B. We found that intra-ACC infusions of NR2B antagonists can block the formation of contextual fear memory, which suggests that cortical NR2B is also involved in the acquisition of contextual memory. Moreover, this finding is consistent with the enhancement of contextual fear memory in transgenic mice overexpressing NR2B in forebrain (Tang et al., 1999). The involvement of ACC in contextual memory acquisition is also in agreement with previous anatomic data that showed the activation of *c-fos* (Morrow et al., 1999) or CREB (Wei et al., 2002) in this area following fear conditioning. Previous studies reported that lesion

in the prefrontal cortex do not impair the acquisition of fear conditioning, which conflicts with the present results (Gewirtz et al., 1997; Morgan and LeDoux, 1999). One major difference is that brain lesions were used in previous studies. In general, brain lesions cause cell/nerve fiber death in the focused area. The effects are also not selective for inhibitory versus excitatory synapses. Furthermore, lesions affect many other nonneuronal cells. We believe that our current study, which combines genetic and pharmacological approaches, avoids many side effects when compared with brain lesioning techniques.

To determine if microinjection of the NR2B antagonist impaired locomotor activity that would interfere with the correct recording of freezing behavior, we measured locomotor activity in an open field after microinjection of Ro25-6981 into the ACC. We found no difference in locomotor activity between mice receiving Ro25-6981 compared to those receiving saline either 15 min after injection or when tested the next day. In addition, we found that there was no significant difference in baseline freezing, freezing during the presentation of the tone, or in immediate freezing, in mice receiving bilateral microinjection of Ro25-6981 into the ACC or hippocampus when compared to animals receiving saline. There was also no significant difference in baseline, tone, or immediate freezing behaviors in mice receiving NR2B siRNA compared to mice receiving only the shock, GFP alone, or control siRNA.

In addition, we examined the contribution of NR2B activation to the acquisition of contextual fear memory in the hippocampus and found that blockade of hippocampal NR2B does not interfere with fear memory, but we cannot conclusively exclude the role of hippocampal NR2B in contextual fear memory by the technique employed (i.e., the drug cannot hit the whole structure, and only a part of the hippocampus might be sufficient to obtain normal performance). Interestingly, the different contribution of NR2B in the ACC and hippocampus to contextual fear memory mimics the differences in LTP mechanisms between these two brain areas; NR2B is involved in LTP induction in the ACC but not in the hippocampus. The behavioral data obtained in the present study are consistent with previous electrophysiological data, which showed that NR2B is not involved in LTP induction in the hippocampus (Liu et al., 2004).

In summary, the present study provides a characterization of NMDA NR2A and NR2B receptors in the ACC LTP. Unlike the hippocampus, NMDA NR2B receptors contribute to synaptic potentiation in the ACC. Our study provides strong evidence that NR2B-containing NMDARs in the ACC can contribute to the formation of classical contextual fear memory. Moreover, based on data available to date, we suggest that the ACC plays an active integrated role in the formation of classic contextual fear memory via anatomic interactions between its neurons and neurons in the amygdala.

### Experimental Procedures

#### Animal

Six- to eight-week-old C57BL/6 male mice and male Sprague Dawley rats were used. All animals were housed under a 12:12 light cycle with food and water provided *ad libitum*. The Animal Care

and Use Committee of the University of Toronto approved the animal protocols.

#### Slice Preparation

Coronal brain slices (300  $\mu\text{m}$ ) from 6- to 8-week-old C57BL/6 mice, containing ACC or hippocampus, were prepared using standard methods (Wei et al., 2001). Slices were transferred to a submerged recovery chamber containing oxygenated (95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ) artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 4.4 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , 25 mM  $\text{NaHCO}_3$ , 1 mM  $\text{NaH}_2\text{PO}_4$ , and 10 mM glucose) at room temperature for at least 1 hr.

#### Whole-Cell Recordings

Experiments were performed in a recording chamber on the stage of an Axioskop 2FS microscope with infrared DIC optics for visualizing whole-cell patch-clamp recordings. EPSCs were recorded from layer II-III neurons using an Axon 200B amplifier (Axon Instruments, CA), and stimulations were delivered using a bipolar tungsten stimulating electrode placed in layer V of the ACC. In hippocampal slices, EPSCs were evoked by stimulating the Schaffer collateral-commissural pathway. EPSCs were induced by repetitive stimulations at 0.02 Hz, and neurons were voltage clamped at  $-70$  mV. The recording pipettes (3–5 M $\Omega$ ) were filled with solution containing 145 mM K-gluconate, 5 mM NaCl, 1 mM  $\text{MgCl}_2$ , 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM  $\text{Na}_3\text{-GTP}$  (adjusted to pH 7.2 with KOH). After obtaining stable EPSCs for 10 min, three kinds of LTP induction paradigms were used within 12 min after establishing the whole-cell configuration to prevent the wash out effect on LTP induction (Tsvetkov et al., 2002). The first protocol involved paired presynaptic 80 pulses at 2 Hz with postsynaptic depolarization at  $+30$  mV (referred to as pairing training). The second involved paired three presynaptic stimuli that caused three EPSPs (10 ms ahead) with three postsynaptic APs at 30 Hz, paired 15 times every 5 s (named the EPSPs-APs protocol). The third involved theta-burst stimulation (five trains of burst with four pulses at 100 Hz, at 200 ms interval; repeated four times at intervals of 10 s; named the TBS). The NMDAR-mediated component of EPSCs was pharmacologically isolated in  $\text{Mg}^{2+}$ -free ACSF containing CNQX (20  $\mu\text{M}$ ), glycine (1  $\mu\text{M}$ ), and picrotoxin (100  $\mu\text{M}$ ). The patch electrodes contained 102 mM cesium gluconate, 5 mM TEA-chloride, 3.7 mM NaCl, 11 mM BAPTA, 0.2 mM EGTA, 30 mM HEPES, 2 mM MgATP, 0.3 mM NaGTP, and 5 mM QX-314 chloride (adjusted to pH 7.2 with CsOH). Neurons were voltage clamped at  $-60$  mV, and NMDAR-mediated EPSCs were evoked at 0.05 Hz. Picrotoxin (100  $\mu\text{M}$ ) was always present to block GABA<sub>A</sub> receptor-mediated inhibitory synaptic currents. The access resistance was 15–30 M $\Omega$  and was monitored throughout the experiment. Data were discarded if access resistance changed by more than 15% during an experiment. Results are expressed as means  $\pm$  SEM. Statistical comparisons were performed using the Student's *t* test.

#### Western Blot and Immunoprecipitation

Equal amounts of protein from the ACC and hippocampus were separated and electrotransferred onto PDVF membranes (Invitrogen), which were probed with anti-NR2A, anti-NR2B (Chemicon), and anti-PSD-95 (ABR) and with  $\beta$ -actin (Sigma) as a loading control. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse protein IgG for PSD-95 and anti-rabbit IgG for the other primary antibodies), and bands were visualized using an ECL system (Perkin Elmer). Synaptosomal membrane fractions (LP1) were prepared as previously described (Dunah and Standaert, 2001) and solubilized using 1% SDS in TEVP buffer: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1X protease inhibitor cocktail (Sigma), and 1X phosphatase inhibitor cocktail 1 and 2 (Sigma). The solubilized proteins were diluted 20-fold with modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF), and incubated with 50  $\mu\text{l}$  of protein G-agarose precoupled with anti-phosphotyrosine antibody (PY20, BD Biosciences) for 3 hr at 4°C. The reaction mixtures were then washed three times and eluted by boiling in sample loading buffer and subjected to Western blot as described above. Equal amounts of synaptosomal membrane fraction from the ACC and hippocampus were used for

the Western blotting of synaptosomal NMDA receptors. Results are expressed as means  $\pm$  SEM. Statistical comparisons were performed using the *t* test or the paired *t* test.

#### Microelectroporation of siRNA

siRNA corresponding to NR2B (sense 5'-GGAUGAGUCCUCCAUGUUCctt-3' and antisense 5'-GAACAUGGAGGACUCAUCctt-3', Silencer Pre-designed siRNA, ID # 61879, Ambion, TX) was delivered into the ACC by microelectroporation, which was performed as described previously (Wei et al., 2003). Briefly, 0.5  $\mu\text{l}$  of 1:4 mixture of siRNA (2.5  $\mu\text{g}/\mu\text{l}$ ) and pEGFP-N1 (1  $\mu\text{g}/\mu\text{l}$ ) was injected into the ACC (0.7 mm anterior to Bregma,  $\pm 0.4$  mm lateral from the midline, 1.8 mm beneath the surface of the skull) (Franklin and Paxinos, 1997) through a 30-gauge injection cannula. Then, square-wave electric pulses (five pulses at 1 Hz, 50 ms duration at 40 V) were delivered through a pair of silver electrodes placed 2 mm anterior and 1 mm posterior to the injection site using a S48 Stimulator (Grass Instruments). Control groups consisted of a group receiving only the electroporation shock, pEGFP-N1, or Silencer Negative control siRNA (#4611, Ambion) and pEGFP-N1. Western blot analysis was performed 4 days after electroporation, and electrophysiological recordings were taken 2–4 days after.

#### Mouse Surgery and Fear Conditioning

Under ketamine and xylazine anesthesia, 24-gauge guide cannulas were implanted bilaterally into the ACC (0.7 mm anterior to Bregma,  $\pm 0.4$  mm lateral from the midline, 1.7 mm beneath the surface of the skull) or dorsal hippocampus (2.0 mm posterior to Bregma,  $\pm 1.5$  mm lateral from the midline, 1.9 mm beneath the surface of the skull). Mice were given at least 2 weeks to recover after cannula implantation. All procedures were performed in accord with the requirements of the Animal Studies Committee at the University of Toronto. The 30-gauge injection cannula was 0.1 mm lower than the guide. For intra-ACC infusion, 0.5  $\mu\text{l}$  Ro25-6981 (4  $\mu\text{g}/\mu\text{l}$ ) or saline was delivered bilaterally within 90 s using a pump. Fifteen minutes later, mice were conditioned by one pairing of a tone (2.8 kHz, 85 dB, 30 s) and a foot shock (0.75 mA, 2 s) that terminated at the same time as the tone. For intrahippocampal infusion, 0.5  $\mu\text{l}$  Ro25-6981 (10  $\mu\text{g}/\mu\text{l}$ ) or saline was delivered bilaterally within 90s. Fifteen minutes later, mice received one pairing of a tone and a foot shock, as above. One day later, animals were exposed to the conditioning context without a tone for 3 min, and freezing responses were scored automatically (Freeze view software, Actriometrics, Wilmette, IL).

#### Rat Surgery and Fear Conditioning

For one-site drug infusion, 23-gauge guide cannulas were implanted bilaterally into the dorsal hippocampus (CA1 region: 3.2 mm posterior to Bregma,  $\pm 1.5$ –1.7 mm lateral from the midline, 1.7 mm beneath the surface of the skull). For two-site drug infusion, guide cannulas were implanted bilaterally into the dorsal hippocampus (first injection site: 2.8 mm posterior to Bregma,  $\pm 1.5$  mm lateral from the midline, 1.7 mm beneath the surface of the skull; second injection site: 3.8 mm posterior to Bregma,  $\pm 1.8$ –2.2 mm lateral from the midline, 1.3 mm beneath the surface of the skull). The dummy cannulas, cut 0.5 mm longer than guide cannulas, were inserted into the guide cannulas to prevent clogging and reduce the risk of infection. Rats were given at least 5 days to recover before experimentation. A 30-gauge injection cannula that was 1.5 mm lower than the guide was used for intrahippocampal infusion. One  $\mu\text{l}$  Ro25-6981 (0.1  $\mu\text{g}/\mu\text{l}$ ) or saline was delivered bilaterally at the rate of 0.5  $\mu\text{l}/\text{min}$  using a pump. After infusion, the cannulas were left in place for an additional 2 min to allow the solution to diffuse away from the cannula tip. Fifteen minutes later, rats were conditioned by one pairing of a tone (2.2 kHz, 96 dB, 30 s) and a foot shock (2.0 mA, 2 s) that terminated at the same time as the tone. Approximately 2 days after conditioning, contextual and auditory memory tests were conducted. Rats were returned to the conditioning chamber and allowed to stay in the chamber for 3 min without footshock.

#### Histological Identification

To confirm the locations of the intra-ACC and hippocampal injection sites, brains were fixed with 4% paraformaldehyde and dehy-

drated through an ascending alcohol series. The mice coronal sections (30  $\mu\text{m}$ ) were mounted on glass slides and stained with hematoxylin and eosin. The rat brains were cut into 40–50  $\mu\text{m}$  coronal sections and stained with neural red. Images were taken using an Olympus light microscope equipped with a CCD camera.

#### Supplemental Data

The Supplemental Data for this article can be found online at <http://www.neuron.org/cgi/content/full/47/6/859/DC1/>.

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