Journal of Cellular Physiology

Long-Term Depression Requires Postsynaptic AMPA GluR2 Receptor in Adult Mouse Cingulate Cortex

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Synaptic long-term depression (LTD) is thought to be important for various brain functions such as learning, memory, and development. Although anterior cingulated cortex (ACC) has been demonstrated to contribute to learning and memory, no studies has been reported about the synaptic mechanisms for cingulate LTD. Here, we used integrative genetic, pharmacological and electrophysiological approaches to demonstrate that AMPA GluR2, but not GluR3, subunit is critical for cingulate LTD. We found that LTD was absent in adult cingulate slices of GluR2 knockout mice. Furthermore, postsynaptic injections of peptides that inhibit AMPA GluR2-PDZ interactions blocked the induction of LTD. To determine if the requirement for AMPA receptor-PDZ interaction is time-dependent, we injected the same inhibiting peptide into the postsynaptic cells 5 min after the induction of LTD. We found that LTD was not affected by the peptide, providing the first evidence that postsynaptic AMPA GluR2-mediated depression occurs rapidly (within $t = 5$ min). Genetic deletion of GluR3 did not affect cingulate LTD. Our results provide the first study of cingulate LTD mechanism using whole-cell patch-clamp recording in adult cortical slices and demonstrate that postsynaptic AMPA GluR2 subunit is crucial for synaptic depression in the ACC of adult mice.

J. Cell. Physiol. 211: 336–343, 2007. © 2006 Wiley-Liss, Inc.

Glutamate activates three different ionotropic receptors in the central nervous system, including receptors for AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate), kainate and NMDA (*N*-methyl-D-aspartate) (Hollmann and Heinemann, 1994). AMPA receptors are heteromeric complexes composed of four distinct subunits GluR1-4 (Hollmann and Heinemann, 1994). AMPA receptor trafficking, which is thought to be critical for long-term potentiation (LTP) and depression (LTD), is regulated by AMPA receptor-PDZ interactions (Daw et al., 2000; Shi et al., 2001; Xia et al., 2000). In the hippocampus, GluR1 subunits are proposed to be rapidly delivered to synapses during LTP (Hayashi et al., 2000; Passafaro et al., 2001). This fast process requires interactions between the GluR1 subunit and the C-terminal of the PDZ protein. In contrast, the GluR2/3 subunits are thought to be important for activity-independent movements of AMPA receptors, which is a constitutive process essential for stable basal synaptic responses (Passafaro et al., 2001; Shi et al., 2001). GluR2/3 subunits are known to interact with PDZ domains of the GRIP (glutamate receptor interacting protein), ABP (AMPA receptor binding protein), and PICK1 (protein interacting with C kinase 1) proteins through their C-terminal sequence (the last four amino acids: SVKI) (Dong et al., 1997; Srivastava et al., 1998; Li et al., 1999; Xia et al., 2000). Previous findings indicate that the C-terminal of the GluR2/3 subunits is involved in hippocampal, cerebellar and spinal plasticity (Daw et al., 2000; Xia et al., 2000; Kim et al., 2001) (see Table 1).

Anterior cingulate cortex (ACC), plays critical roles in learning, memory, pain, and emotion in humans and animals (Frankland et al., 2004; Maviel et al., 2004; Wiltgen et al., 2004; Tang et al., 2005; Zhao et al., 2005). Recent studies indicate that excitatory synaptic transmission in the ACC is mostly mediated by AMPA receptors (Wu et al., 2005b); and synaptic LTP and LTD can be observed in in vitro brain slices and in in vivo whole animals (Gorkin et al., 2003; Zhuo, 2004; Toyoda et al., 2005; Zhao et al., 2005). Peripheral injury activates immediate early genes in the ACC and affected cingulate LTD in adult animals (Wei et al., 1999). Furthermore, using in vivo field or intracellular recording approaches, long-term plastic changes in excitatory transmission in the ACC are observed in response to various physiological and pathological manipulations (Wei et al., 1999; Wei and Zhuo, 2001; Wu et al., 2005a), suggesting that cingulate plasticity including LTP and LTD may play important roles in ACC-related brain functions.

Considering the potential importance of cingulate cortical LTD, it is important to dissect the signaling pathways underlying synaptic depression in this region. Previous studies of the roles of AMPA receptor subunits in synaptic depression were mainly focused on the hippocampus and cerebellum (Daw et al., 2000; Xia et al., 2000; Chung et al., 2003; Meng et al., 2003; Steinberg et al., 2004). In the ACC, no study has been reported about the

Hiroki Toyoda, Long-Jun Wu and Ming-Gao Zhao contributed equally to this manuscript.

Contract grant sponsor: The Canadian Institutes of Health Research.
Contract grant sponsor: The EJLB-CIHR Michael Smith Chair.
Contract grant sponsor: The Canada Research Chair.
Contract grant sponsor: The Canadian Institutes of Health Research and Fragile X Research Foundation of Canada.

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Received 15 August 2006; Accepted 13 October 2006

DOI: 10.1002/jcp.20940

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TABLE 1. Function of AMPA receptor subunits and interacting proteins in LTD

Brain region	AMPA receptors and protein	Effect on LTD	References
Hippocampus	GluR2/3 double knockout mice	No effect	Meng et al. (2003)
	GluR2 knockout mice	No effect	Meng et al. (2003)
	GluR3 knockout mice	No effect	Meng et al. (2003)
	GluR2-NSF peptide	Blocked	Luscher et al. (1999); Luthi et al. (1999); Lee et al. (2002)
	GluR2-GRIP/ABP peptide	Blocked	Daw et al. (2000); Kim et al. (2001)
	GluR2-PICK1 peptide	No effect	Daw et al. (2000)
		Blocked	Kim et al. (2001)
Cerebellum	GluR2 knockout mice	Blocked	Chung et al. (2003)
	GluR2-NSF peptide	Blocked	Steinberg et al. (2004)
	GluR2-GRIP/ABP peptide	Blocked	Xia et al. (2000)
	GluR2-PICK1 peptide	Blocked	Xia et al. (2000)
	PICK1 knockout mice	Blocked	Steinberg et al. (2006)
ACC	GluR2 knockout mice	Blocked	This study
	GluR2-GRIP/ABP peptide	Blocked	This study
	GluR2-PICK1 peptide	Blocked	This study
	GluR3 knockout mice	No effect	This study

possible role for AMPA receptor subunits and AMPA receptor-interacting proteins for the induction of LTD. In the present study, we employed integrative approaches, including knockout mice, electrophysiological recording and postsynaptic injection of inhibiting peptide, to investigate if the induction or expression of LTD in the ACC requires AMPA GluR2 and/or GluR3 subunits.

Materials and Methods

Animals

GluR2$^{-/-}$ and *GluR3*$^{-/-}$ mice were provided by Dr. Zhengping Jia and maintained in University of Toronto animal facility (Jia et al., 1996; Meng et al., 2003). Littermate wild-type *GluR2*$^{+/+}$ and *GluR3*$^{+/+}$ animals were used as controls. In some experiments that are not related to gene knockout mice, adult male C57BL/6 mice purchased from Charles River were used. All mice were maintained on a 12 h light/dark cycle withfood and water provided ad libitum. The Animal Studies Committee at the University of Toronto approved all experimental protocols.

Slice preparation

Coronal brain slices (300 μm) containing the ACC from 6- to 8-week-old wild-type and GluR2 or 3 knockout mice were prepared using standard methods (Zhao et al., 2005). Slices were transferred to a submerged recovery chamber with oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM: 124 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 25 $NaHCO_3$, 1 NaH_2PO_4, 10 glucose) at room temperature for at least 1 h.

Whole-cell patch clamp recordings

Experiments were performed in a recording chamber on the stage of an Axioskop 2FS microscope with infrared DIC optics for visualization of whole-cell patch clamp recording. Excitatory postsynaptic currents (EPSCs) were recorded from layer II/III neurons with an Axon 200B amplifier (Molecular devices, CA) and the stimulations were delivered by a bipolar tungsten stimulating electrode placed in layer V of the ACC slices. Neurons in layer II and III receive inputs from medial thalamus (Wang and Shyu, 2004). EPSCs were induced by repetitive stimulations at 0.02 Hz and neurons were voltage clamped at −70 mV. The recording pipettes (3–5 MΩ) were filled with solution containing (mM): 145 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.1 Na_3-GTP (adjusted to pH 7.2 with KOH).
The LTD and LTP induction paradigm was used within 12 min after establishing the whole-cell configuration to prevent wash out effect on LTD induction (Toyoda et al., 2005). LTD was induced by 300 pulses at 1 Hz paired with postsynaptic depolarization at −45 mV (also referred to as "paired training"). The LTP induction protocol involved paired presynaptic 80 pulses at 2 Hz with postsynaptic depolarization at +30 mV (Zhao et al., 2005). NMDA receptor-mediated component of EPSCs was pharmacologically isolated in ACSF containing: CNQX (20 μM), glycine (1 μM) and picrotoxin (100 μM). The patch electrodes for NMDA receptor-mediated EPSCs contained (in mM) 102 cesium gluconate, 5 TEA chloride, 3.7 NaCl, 11 BAPTA, 0.2 EGTA, 20 HEPES, 2 MgATP, 0.3 NaGTP, and 5 QX-314 chloride (adjusted to pH 7.2 with CsOH). Neurons were voltage clamped at −30 mV and NMDA receptor-mediated EPSCs were evoked at 0.05 Hz. Picrotoxin (100 μM) was always present to block $GABA_A$ receptor-mediated inhibitory currents and monitored throughout the synaptic currents. In some experiments, we included spermine (100 μM) in the internal solution to test the I–V relationship of AMPA receptor-mediated EPSCs (Bowie and Mayer, 1995; Donevan and Rogawski, 1995; Kamboj et al., 1995). Access resistance was 15–30 MΩ and was monitored throughout the experiment. Data were discarded if access resistance changed more than 15% during an experiment. Statistical comparisons were performed using the Student's *t*-test.

Intracellular application of a drug

The GluR2 C-terminal peptide was injected through a perfusion tube directly installed in a postsynaptic patch pipette. After back-filling the tube with intracellular solutions containing one of these peptides, it was inserted into a patch pipette with its tip 500 μm behind the pipette tip. At 5 min after LTD induction, the peptide was delivered into a postsynaptic patch pipette with positive pressure manually applied through a syringe (Hori et al., 1999) (Fig. 7A). When Lucifer yellow (0.1%) was injected by this method, fluorescence was detected in an ACC neuron within 1 min after injection and reached maximal intensity within 5 min.

Pharmacological inhibitors

All chemicals and drugs were obtained from Sigma (St. Louis, MO), except for Pep2-SVKE, Pep2-SVKI, Pep2-AVKI, and QX-314, which were from Tocris Cookson (Ellisville, MO). The selection of the inhibitors and doses are in part based on previous reports (Li et al., 1999; Wei et al., 1999; Zhao et al., 2005; Wu et al., 2005a).

Data analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed with the Student's *t*-test or one-way analysis of variance (ANOVA). In all cases, $P < 0.05$ was considered statistically significant.

Results

To investigate the role of GluR2 and GluR3 subunits in the induction of LTD, we performed conventional whole-cell patch-clamp recordings from visually identified pyramidal neurons in layer II/III of ACC slices. Fast EPSCs were obtained by delivering focal electrical stimulation to layer V (Fig. 1A). We recorded EPSCs from neurons in layers II-III of the ACC, because layer II–III pyramidal neurons are known to receive projections from medial thalamus (Wang and Shyu, 2004), and the superficial layers of the ACC receive substantial glutamate

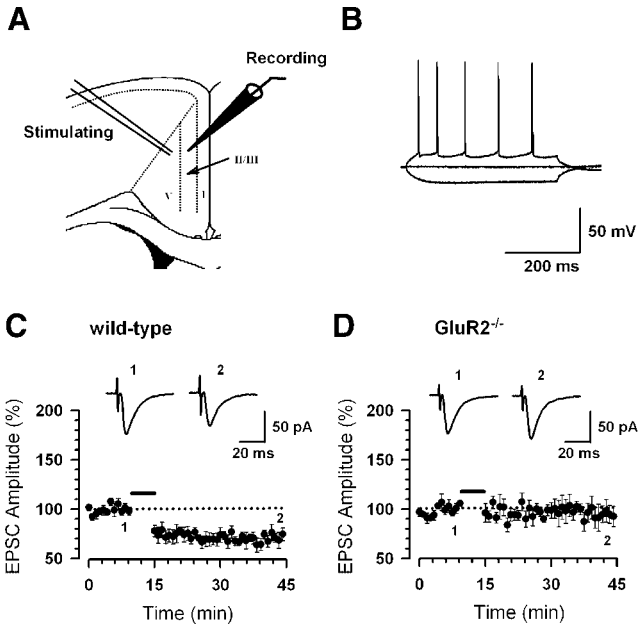

Fig. 1. Synaptic depression in GluR2 knockout mice. A: Diagram of a slice showing the placement of a whole-cell patch recording and stimulation electrode in the ACC slice. B: This trace shows typical voltage responses to current injections of −100, 0, and 100 pA in a ACC neuron from a wild-type mouse. C: LTD is induced in ACC neurons in wild-type mice (n = 7 slices/6 mice). D: LTD is absent in ACC neurons in GluR2 knockout mice (*GluR2*$^{-/-}$) (n = 9 slices/6 mice). C, D: The insets show averages of six EPSCs at baseline response (1) and 30 min (2) after the paired training (bar). The dashed line indicates the mean basal synaptic response.

input from layer V (Wei et al., 1999). AMPA receptors are composed of different combinations of GluR1-4 subunits (Hollmann and Heinemann, 1994). In the adult hippocampus, most AMPA receptors are composed of GluR1/2 or GluR2/3 heteromers (Wenthold et al., 1996). Previous reports using both immunostaining and in situ hybridization showed that GluR1, GluR2 and GluR3 are enriched in the layer II–III in the ACC (Sato et al., 1993) and intense GluR2/3 immunostaining was expressed in ACC pyramidal neurons in layer II/III (Wei et al., 1999). Therefore, fast EPSCs in the present study appear to be mediated by GluR1/2 or GluR2/3 heteromers, since most AMPA receptos exist as heteromeric assemblies of GluR1/2 or GluR2/3 (Wenthold et al., 1996). In addition to visual identification by putting Lucifer yellow into the intracellular solution (Zhao et al., 2005), we confirmed that the recordings were performed from ACC pyramidal cells by injecting depolarizing currents into the neuron. Injection of depolarizing currents into neurons induced repetitive action potentials with frequency adaptation that is typical of the firing pattern of pyramidal neurons (Fig. 1B).

GluR2 subunit contributes to the induction of cingulate LTD

Although the subunit-specific role of GluR2 and GluR3 in LTD induction has been studied in the hippocampus (Meng et al., 2003), little is known about the role of these subunits in cingulate LTD of adult mice. To study the subunit-specific function of GluR2 and GluR3 in cingulate LTD, we examined synaptic depression in ACC neurons from GluR2 or GluR3 knockout mice (*GluR2*$^{-/-}$ or *GluR3*$^{-/-}$). First, we investigated the role of GluR2 subunit in the induction of ACC synaptic depression. We have previously shown that pairing presynaptic stimulation with postsynaptic depolarization (or called paired training) (300 pulses of presynaptic stimulation at 1 Hz in layer V with postsynaptic depolarization at −45 mV) induced long-term depression in cingulate slices (Toyoda et al., 2005). LTD was induced with paired training within 12 min after establishing the whole-cell configuration to avoid washout of intracellular contents that are critical for the establishment of synaptic plasticity (Ko et al., 2005; Zhao et al., 2005). All baseline amplitude of EPSC was adjusted to 50–100 pA before LTD induction. Synaptic depression in *GluR2*$^{-/-}$ mice was completely abolished (93.9 ± 10.6% of baseline response, n = 9 slices/6 mice; $P > 0.05$ compared with baseline response, Fig. 1D), while LTD was induced in wild-type controls (71.9 ± 6.3% of baseline response, n = 7 slices/6 mice; $P < 0.05$ compared with baseline response before the stimulation, Fig. 1C). These results suggest that the GluR2 contributes to the induction of cingulate LTD.

We further examined LTP in the *GluR2*$^{-/-}$ mice. The LTP induction protocol involved paired presynaptic 80 pulses at 2 Hz with postsynaptic depolarization at +30 mV (Zhao et al., 2005). The protocol produced LTP in *Glu2*$^{-/-}$mice (175.0 ± 10.4%, n = 9 slices/5 mice; $P < 0.05$ compared with baseline responses). The magnitude of synaptic potentiation in *GluR2*$^{-/-}$ mice was significantly greater than that of littermate wild-type mice (149.1 ± 11.2% of baseline, n = 8 slices/5 mice, *t*-test; $P < 0.05$ compared with baseline responses, $P < 0.01$ compared to slices of *Glu2*$^{-/-}$mice).

Synaptic transmission and paired-pulse facilitation (PPF) in GluR2 knockout mice

To further explore the effect of GluR2 deletion on cingulate excitatory synaptic transmission and plasticity, we measured AMPA receptor mediated basal synaptic transmission and paired-pulse facilitation (PPF). First, experiments were performed to examine whether basic synaptic transmission was altered in *GluR2*$^{-/-}$ mice. We analyzed AMPA receptor-mediated EPSCs evoked by stimulation at various intensities. The input-output relationship of AMPA receptor-mediated EPSCs in *GluR2*$^{-/-}$ mice (n = 7) was significantly reduced as compared with their littermate wild-type mice (n = 8) (Fig. 2A). We further analyzed the kinetics of AMPA EPSCs in *GluR2*$^{-/-}$ mice. Our results showed that there is no significant difference in either rise time (10%–90% of peak amplitude, in ms: wild-type, 3.1 ± 0.1; *GluR2*$^{-/-}$, 3.2 ± 0.1, $P > 0.05$) or the decay time constant (in ms: wild-type, 17.4 ± 0.5; *GluR2*$^{-/-}$, 18.4 ± 0.8, $P > 0.05$) between wild-type and *GluR2*$^{-/-}$ mice. Considering the important roles of NMDA receptor in the induction of cingulate LTD ((Toyoda et al., 2005), we next examined if NMDA receptor mediated responses may be altered. NMDA receptor mediated responses were recorded after the blockade of AMPA/kainate receptor mediated responses by CNQX (20 μM). We found that NMDA receptor-mediated EPSCs were not affected in *GluR2*$^{-/-}$ knockout mice (n = 7) as compared with wild-type mice (n = 6) (Fig. 2B).

To test whether presynaptic function was altered in *GluR2*$^{-/-}$ knockout mice, we measured paired-pulse facilitation (PPF) in slices of wild-type and knockout mice. We found that there was no significant difference in the PPF between wild-type (n = 9) and *GluR2*$^{-/-}$ mice (n = 10) (Fig. 2C), indicating that presynaptic function is not altered.

Voltage dependence of AMPA and NMDA receptor-mediated currents

To examine the voltage dependence of AMPA and NMDA receptor-mediated currents, we recorded synaptic responses

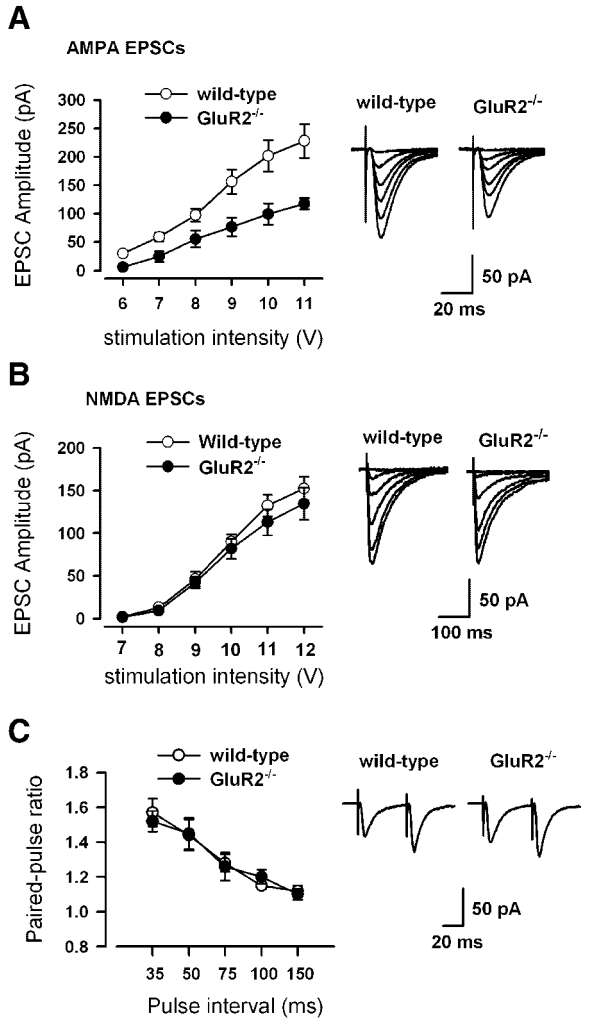

Fig. 2. Synaptic transmission in GluR2 knockout mice. A: Input-output relationship for AMPA receptor-mediated EPSCs in GluR2 knockout (*GluR2*$^{-/-}$) (n = 7) and wild-type (n = 8) mice. Input-output relationship for AMPA receptor-mediated EPSCs in *GluR2*$^{-/-}$ mice is significantly decreased as compared with wild-type mice. B: Input–output relationship for NMDA receptor-mediated EPSCs in GluR2 knockout (*GluR2*$^{-/-}$) (n = 7) and wild-type (n = 6) mice. C: Paired-pulse facilitation (PPF) does not differ in *GluR2*$^{-/-}$ (n = 10) and wild-type (n = 9) mice. Sample traces of PPF recorded from *GluR2*$^{-/-}$ and wild-type mice (at the 50 msec interval).

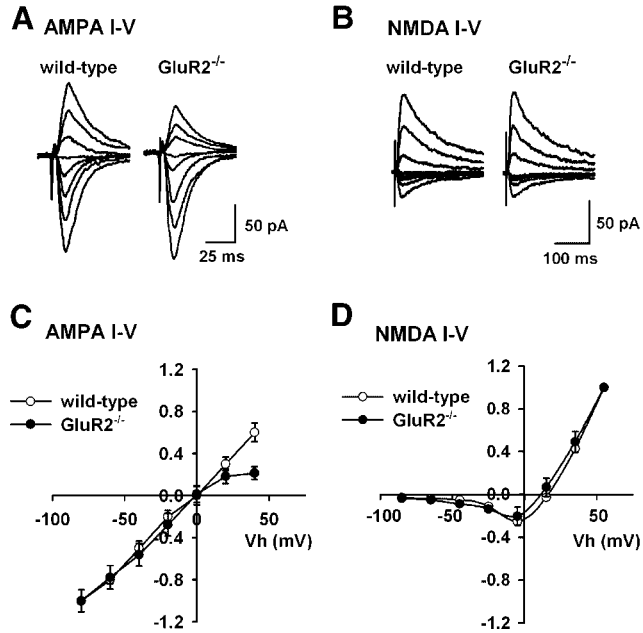

Fig. 3. Current–voltage relationship of the AMPA and NMDA receptor-mediated EPSCs. A: Sample traces of AMPA receptor-mediated EPSCs evoked at different holding potentials from GluR2 knockout (*GluR2*$^{-/-}$) and wild-type mice. B: Sample traces of NMDA receptor-mediated EPSCs evoked at different holding potentials from *GluR2*$^{-/-}$ and wild-type mice. C: Normalized current-voltage plots for AMPA receptor-mediated EPSCs between *GluR2*$^{-/-}$ (n = 7) and wild-type mice (n = 6). AMPA currents from *GluR2*$^{-/-}$ mice show a significant rectification at the positive holding potentials. AMPA receptor-mediated EPSCs were normalized to the EPSC at −80 mV. D: Normalized current–voltage plots for NMDA receptor-mediated EPSCs between *GluR2*$^{-/-}$ (n = 8) and wild-type mice (n = 7). Normalized current-voltage relationship for NMDA receptor-mediated EPSCs in *GluR2*$^{-/-}$ mice was similar to those in wild-type mice. NMDA receptor-mediated EPSCs were normalized to the EPSC at +55 mV.

in voltage-clamp mode, respectively. For AMPA receptor mediated responses, AMPA currents from wild-type mice (n = 6) were linear, while those from *GluR2*$^{-/-}$ mice (n = 7) showed a significant rectification at the positive holding potentials (with spermine (100 μM) in the internal solution) (Fig. 3A and C). The reduction of outward currents at positive holding potentials is consistent with previous reports (Hollmann et al., 1991; Geiger et al., 1995).

For NMDA receptor mediated responses, we first isolated NMDA receptor mediated responses as described above. We then measured NMDA receptor mediated EPSCs at different holding potentials from wild-type and *GluR2*$^{-/-}$ knockout mice. We found that the I/V relationship of the NMDA component remained unchanged in *GluR2*$^{-/-}$ mice (n = 8) as compared that from wild-type mice (n = 7) (Fig. 3B,D).

AMPA GluR2 and PDZ interaction is required for cingulate LTD

To test if the interaction between the GluR2/3 C-terminal and its specific PDZ binding partners is important for the induction of LTD, we used several synthetic peptides that disrupt the interaction between AMPA subunits and PDZ-containing proteins. Peptides were applied through the patch recording electrode into postsynaptic neurons. First, we used a control peptide (Pep2-SVKE), in which the PDZ interaction motif is inhibited by substituting the last amino acid (isoleucine) with glutamate (Li et al., 1999). Postsynaptic application of Pep2-SVKE (100 μM) did not affect basal synaptic transmission in ACC slices (last 5 min, 98.7 ± 6.1% of first 5 min baseline response, n = 7, $P > 0.05$, Fig. 4A). In the presence of Pep2-SVKE, LTD was not affected (mean 71.9 ± 5.2% of baseline response, n = 8, $P < 0.05$ compared with baseline response before the paired training, Fig. 4B). Next, we tested a Pep2-SVKI peptide, which interferes with interactions between GluR2 and GRIP (glutamate receptor interaction protein), ABP (AMPA receptor binding protein) and PICK1 (Li et al., 1999; Daw et al., 2000; Xia et al., 2000; Kim et al., 2001). In the presence of Pep2-SVKI (100 μM), basal synaptic responses were also unchanged (last 5 min, 103.4 ± 7.2% of first 5 min baseline response, n = 6, $P > 0.05$, Fig. 4C). However, LTD was completely blocked by the presence of Pep2-SVKI (94.6 ± 5.5%

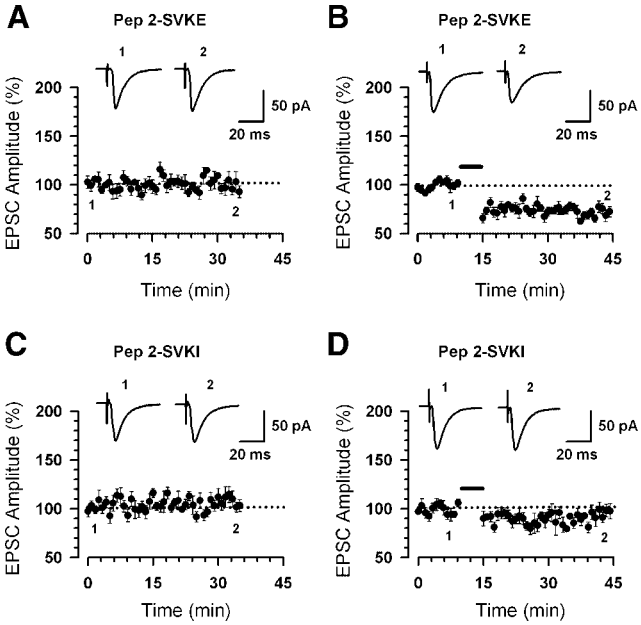

Fig. 4. Pep2-SVKI blocks the induction of LTD. A, C: Pep2-SVKE (100 μM) and Pep2-SVKI (100 μM) do not affect baseline response. The insets show averages of six EPSCs at the time points of 5 (1) and 35 min (2) during the recording. The dashed line indicates the mean basal synaptic response. B: Pep2-SVKE (100 μM, n = 8) has no effect on the induction of LTD. D: In the presence of Pep2-SVKI (100 μM) in the intracellular solution, LTD is blocked (n = 10). B and D: Traces show averages of six EPSCs at baseline responses (1) and 30 min (2) after the paired training (bar). The dashed line indicates the mean basal synaptic response.

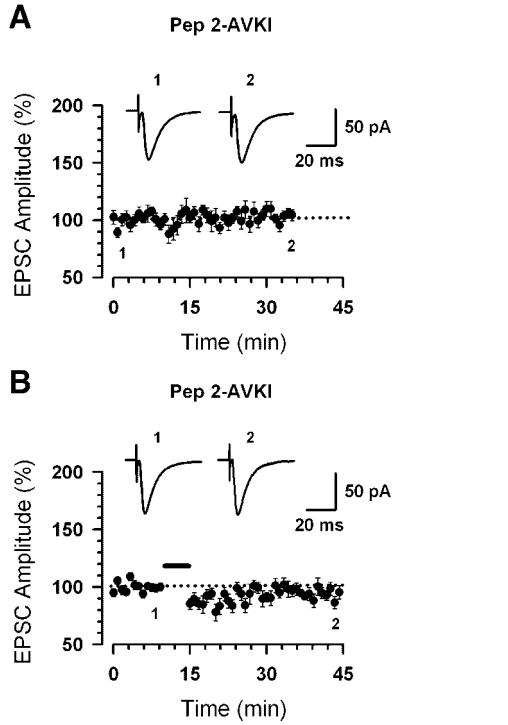

Fig. 5. Pep2-AVKI blocks the induction of LTD. A: Pep2-AVKI (100 μM) does not affect baseline response. The insets show averages of six EPSCs at the time points of 5 (1) and 35 min (2) during the recording. The dashed line indicates the mean basal synaptic response. B: In the presence of Pep2-AVKI (100 μM) in the intracellular solution, LTD is blocked (n = 6). B: Traces show averages of six EPSCs at baseline responses (1) and 30 min (2) after the paired training (bar). The dashed line indicates the mean basal synaptic response.

of baseline response, n = 10, $P > 0.05$ compared with baseline response before the paired training, Fig. 4D). These results provide the direct evidence that postsynaptic interaction between AMPA GluR2 receptor and PDZ protein is critical for the induction of cingulate LTD.

We then used a Pep2-AVKI peptide, which disrupts binding of GluR2 to PICK1 (protein interaction with C kinase) (Li et al., 1999; Daw et al., 2000; Xia et al., 2000; Kim et al., 2001). We found that postsynaptic application of Pep2-AVKI (100 μM) had no effect on basal synaptic transmission (last 5 min, 99.5 ± 3.8% of first 5 min baseline response, n = 7, $P > 0.05$, Fig. 5A). We then investigated the effects of Pep2-AVKI on LTD and found that LTD was blocked by the presence of Pep2-AVKI (95.5 ± 7.2% of baseline response, n = 6, $P > 0.05$ compared with baseline response before the paired training, Fig. 5B). These results strongly suggest that both GluR2/3-PICK1 and GluR2/3-GRIP1/ABP interactions are likely contribute to cingulate LTD.

NMDA mediated responses are not affected

Cingulate LTD requires activation of postsynaptic NMDA receptors (Toyoda et al., 2005). To exclude the possible inhibition of NMDA receptors by these peptides, we measured the effects of postsynaptic injection of the peptides on NMDA receptor-mediated EPSCs. We found that these peptides had no effect on NMDA receptor mediated EPSCs (Pep2-SVKE: last 5 min mean 99.1 ± 5.8% of first 5 min baseline response, n = 5, $P > 0.05$; Pep2-SVKI: last 5 min mean 101.3 ± 5.3% of first 5 min baseline response, n = 6, $P > 0.05$; Pep2-AVKI: last 5 min mean 103.7 ± 4.0% of first 5 min baseline response, n = 6, $P > 0.05$) (Fig. 6), indicating that the blocking effects are not simply due to the inhibition of NMDA receptor functions in the cingulate neurons.

Requirement of GluR2 PDZ interaction is time-dependent

In this study, we demonstrated that GluR2 subunit is essential for the induction of LTD, however, how GluR2 subunit contributes to the expression of LTD remains unknown. To examine the role of GluR2 subunit in the expression of LTD, we injected Pep2-SVKI into neurons 5 min after LTD induction (Hori et al., 1999) (Fig. 7A). In comparison with application before the induction, we found no significant effect on the expression of LTD during the 25 min treatment with Pep2-SVKI (83.3 ± 7.8% at 25 min post paired training, 20 min Pep2-SVKI treatment, n = 5, $P < 0.05$ compared with baseline response before the paired training, Fig. 7B). Although we cannot rule out the possibility that Pep2-SVKI perfusion is not efficient than Lucifer yellow due to a large molecule of Pep2-SVKI, the maintenance phase of LTD was not affected by Pep2-SVKI. This result suggests that GluR2 receptor-mediated possible trafficking events are completed within 5–10 min after LTD induction.

Synaptic plasticity and transmission in GluR3 knockout mice

We then examined the possibility that GluR3 may play a similar role in synaptic depression, since GluR3 is closely related to GluR2 in the structure and biochemical properties (Hollmann

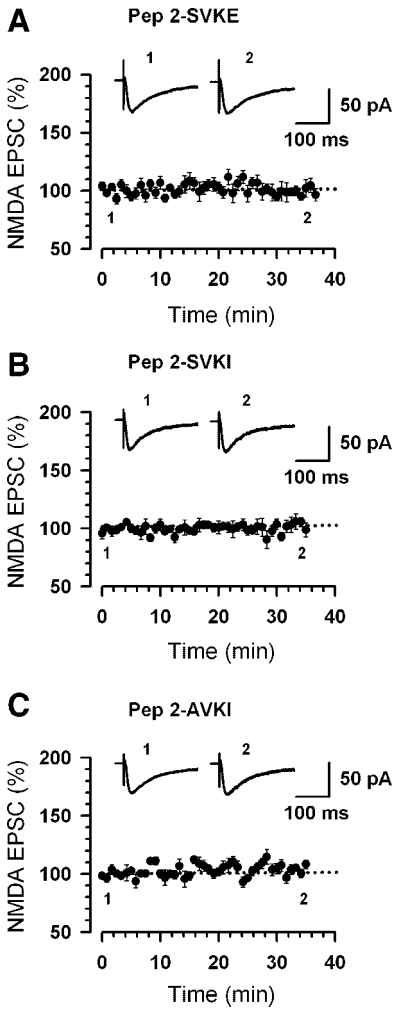

Fig. 6. GluR2 C-terminal peptides have no effect on NMDA receptor-mediated EPSCs. A–C: Postsynaptic injection of Pep2-SVKE (100 μM, n = 5), Pep2-SVKI (100 μM, n = 6), or Pep2-AVKI (100 μM, n = 6) has no effect on NMDA receptor-mediated EPSCs. The insets show averages of six NMDA receptor-mediated EPSCs at the time points of 5 (1) and 35 min (2) during the recording. The dashed line indicates the mean basal synaptic response.

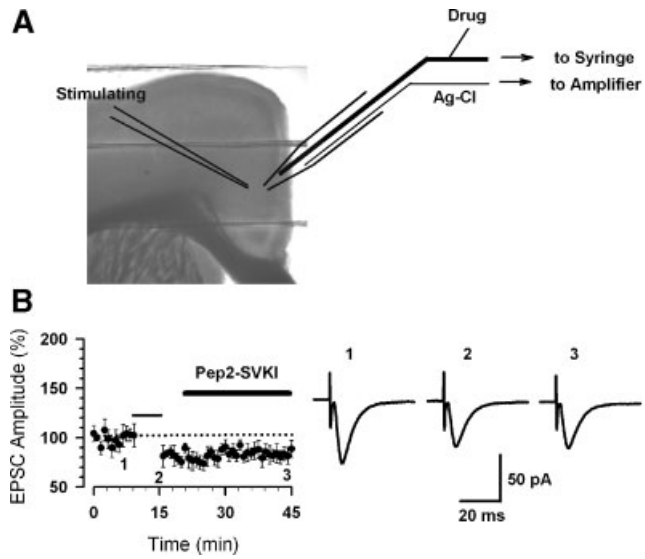

Fig. 7. GluR2 PDZ interaction is rapidly completed. A: Diagram of a slice showing the placement of an intracellular application system and a stimulation electrode in a ACC slice. B: Direct perfusion of Pep2-SVKI (100 μM, n = 5) 5 min after paired training has no effect on the maintenance of LTD. Traces show averages of six EPSCs at baseline response (1) and 5 min (2) and 30 min (3) after the paired training (bar). The dashed line indicates the mean basal synaptic response.

and Heinemann, 1994). We tested LTD in ACC slices from *GluR3*$^{-/-}$ and wild-type mice and found that LTD was comparable in both groups (wild-type, 73.5 ± 4.1% of baseline response, n = 6 slices/5 mice; $P < 0.05$ compared with baseline response, Fig. 8A: *GluR3*$^{-/-}$, 74.9 ± 6.2% of baseline response, n = 8 slices/5 mice; $P < 0.05$ compared with baseline response, Fig. 8B). To further examine the role of GluR3, we recorded synaptic transmission and PPF. There was no difference in the input-output relationship of AMPA receptor-mediated EPSCs between *GluR3*$^{-/-}$ (n = 6) and wild-type mice (n = 6) (Fig. 8C). Similarly, no difference was found in the PPF between *GluR3*$^{-/-}$ (n = 7) and wild-type mice (n = 7) (Fig. 8D). Then, we examined the voltage dependence of AMPA and NMDA receptor-mediated currents in *GluR3*$^{-/-}$ and wild-type mice. The I/V relationship of the AMPA and NMDA component was not significantly different in *GluR3*$^{-/-}$ and wild-type mice, respectively (AMPA I/V relationship: *GluR3*$^{-/-}$, n = 6, wild-type, n = 6; NMDA I/V: *GluR3*$^{-/-}$, n = 6, wild-type, n = 7) (Fig. 8E,F).

Taken together, synaptic depression was not affected by the deletion of GluR3 subunit.

Discussion

Using integrative approaches, we show here that GluR2 subunit plays key roles in the induction of LTD in the ACC. Moreover, we show that the possible trafficking of GluR2 subunit after LTD induction is completed within 5–10 min. These results suggest that after the induction of LTD, GluR2 containing receptors are removed from ACC synapses and such delivery events are completed in a rapid time scale. Considering the important roles of the ACC in various physiological and pathological functions, we believe that the present study is novel for our understanding of molecular and synaptic mechanisms of prefrontal cortical functions. Our results confirm and extend the previous work in the hippocampus and other brain areas that AMPA GluR2 receptor play critical roles in synaptic depression.

Synaptic transmission and plasticity in the ACC

Glutamate is the major fast excitatory transmitter in the ACC. Different types of glutamate receptors, including AMPA, KA, NMDA, and metabotropic glutamate receptors (mGluRs), are found in the ACC (Zhuo, 2004). Moreover, electrophysiological studies have shown that all three ionotropic glutamate receptors contribute to excitatory postsynaptic current in acute ACC slices, and AMPA receptors mediate most of postsynaptic responses (Wei et al., 1999; Wu et al., 2005a,b). Our recent studies demonstrate that glutamatergic synapses in the ACC can undergo long term plastic changes, both LTP and LTD. NMDA receptor and its downstream molecules as AC1, CaMKIV are required for the induction of cingulate LTP (Wei et al., 2002; Liauw et al., 2005; Zhao et al., 2005). Prolonged, low frequency stimulation produced LTD in ACC slices by using field recording in adult cingulate slices (Wei et al., 1999; Wu et al., 2005b). mGluRs and

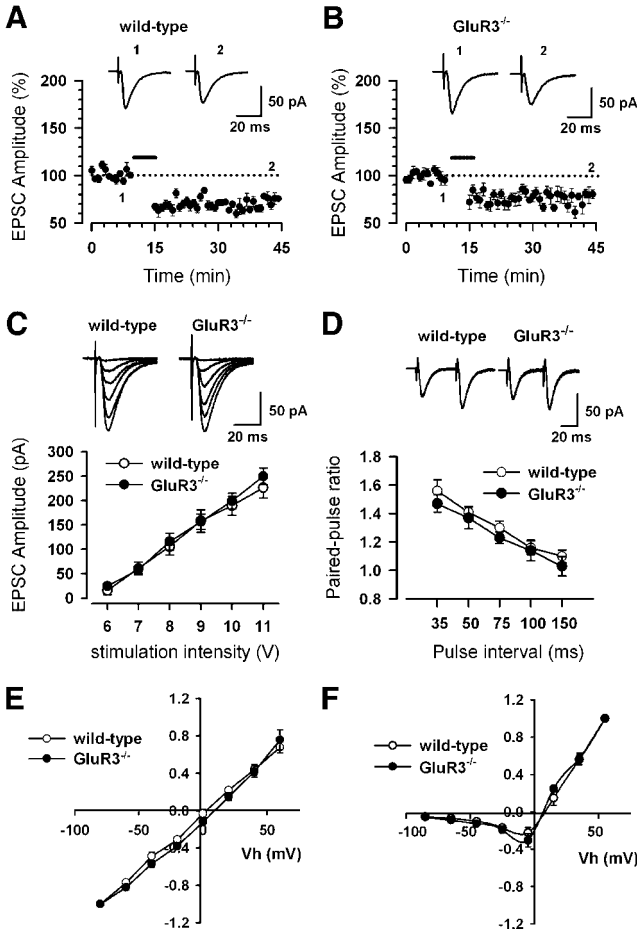

Fig. 8. Synaptic depression and transmission in GluR3 knockout mice. A, B: LTD is observed in ACC neurons in wild-type (n = 6 slices/5 mice) and GluR3 knockout (*GluR3*$^{-/-}$) mice (n = 8 slices/5 mice). The insets show averages of six EPSCs at baseline response (1) and 30 min (2) after the paired training (bar). The dashed line indicates the mean basal synaptic response. C: Input–output relationship for AMPA receptor-mediated EPSCs shows no difference between wild-type (n = 6) and *GluR3*$^{-/-}$ mice (n = 6). The insets show averages of six AMPA receptor-mediated EPSCs evoked with various stimulus intensities. D: Paired-pulse facilitation (PPF) shows no difference between wild-type (n = 7) and *GluR3*$^{-/-}$ mice (n = 7). Sample traces of PPF recorded from *GluR3*$^{-/-}$ and wild-type mice (at the 50 msec interval). E: Normalized current-voltage plots for AMPA receptor-mediated EPSCs are comparable between wild-type (n = 6) and *GluR3*$^{-/-}$ mice (n = 6). AMPA receptor-mediated EPSCs were normalized to the EPSC at −80 mV. F: Normalized current-voltage plots for NMDA receptor-mediated EPSCs show no change between wild-type (n = 7) *and GluR3*$^{-/-}$ mice (n = 6). NMDA receptor-mediated EPSCs were normalized to the EPSC at +55 mV.

L-type voltage-gated calcium channels (Wei et al., 1999) or NMDA receptors (Toyoda et al., 2005) are required for the induction of LTD. Under whole-cell patch-clamp recording condition in adult cingulate slices, the induction of LTD is NMDA receptor dependent (Toyoda et al., 2005). These findings suggest that different postsynaptic receptor or channels may be employed to the LTD induction depending on the induction protocols and/or recording methods.

GluR2 and LTD

GluR2/3 subunits may continually replace preexisting synaptic AMPA receptors in an activity-independent manner (Carroll et al., 2001; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003) and play a complementary role via GluR2-mediated interactions with NSF (*N*-ethylmaleimide sensitive fusion protein), AP2 (adaptor protein complex 2) (Nishimune et al., 1998; Song et al., 1998; Lee et al., 2002), and PDZ proteins such as GRIP1, ABP, and PICK1 (Dong et al., 1997; Daw et al., 2000; Kim et al., 2001). In the cerebellum, GluR2 interactions with NSF (Steinberg et al., 2004) and PDZ domain proteins GRIP/ABP and PICK1 (Xia et al., 2000) are suggested to be required for LTD induction. Cerebellar LTD was absent in mice lacking the GluR2 subunit, and the phosphorylation of Serine 880 of GluR2 plays a key role for cerebellar LTD (Chung et al., 2003). Furthermore, cerebellar LTD was completely abolished in PICK1 knockout mice, suggesting the importance of the GluR2-PICK1 interaction for cerebellar LTD expression (Steinberg et al., 2006). In the hippocampus, GluR2-NSF interactions were found to be important for synaptic depression (Luscher et al., 1999; Luthi et al., 1999), and the phosphorylation of Serine 880 within the C terminal PDZ ligand of GluR2 was also shown to be critical for LTD induction (Kim et al., 2001). Thus, the interactions of the GluR2 C-terminal tail with several intracellular proteins such as GRIP1, ABP, and PICK1 could be an important mechanism for the induction of LTD in the cerebellum and hippocampus. In the present study, we injected GluR2 synthetic peptides into postsynaptic neurons to investigate the role of the GluR2 subunit in cingulate LTD. Although previous studies reported that the interfering peptides (Pep2-AVKI and/or Pep2-SVKI) caused changes in basal synaptic transmission (Daw et al., 2000; Kim et al., 2001), we found that the peptides (Pep2-AVKI and Pep2-SVKI) did not affect basal synaptic transmission in the ACC slices. Several reasons, such as different brain regions, the age of animals, and the different experimental conditions might have caused this discrepancy. Moreover, intracellular perfusion of GluR2 peptides, both Pep2-SVKI and Pep2-AVKI, completely blocked cingulate LTD. These results suggest that cingulate LTD shares a similar mechanism with the hippocampus and cerebellum, in which interactions of the GluR2 C-terminal PDZ proteins with GRIP1, ABP and PICK1 are essential for LTD induction (Xia et al., 2000; Kim et al., 2001).

The subunit-specific function of GluR2 and/or GluR3 in hippocampal LTD has been investigated in mice lacking GluR2 and/or GluR3 subunits (Jia et al., 1996; Meng et al., 2003). These studies indicate that GluR2 and/or GluR3 subunits are not necessary for the induction of LTD, although how LTD is induced in these mutant mice remains unclear. However, we demonstrate here that LTD was absent in mice lacking GluR2, but not GluR3 subunits, indicating that the GluR2-containing AMPA receptor contributes to the induction of LTD in the ACC. Because of the lack of selective antagonists against GluR1/2 heteromers, we cannot rule out the possibility that GluR1/2 heteromers may also contribute to LTD in the ACC. Future experiments will be needed when such selective antagonist become available. Therefore, both GluR1/2 and GluR2/3 heteromers may involve in LTD in the ACC. In contrast to GluR2 subunits, the functional role of GluR3 subunits remains uncertain. Our preliminary data of GluR3 knockout mice showed that GluR3 deletion affect behavioral emotional responses (unpublished data), indicating that GluR3 subunit may also important roles in high brain functions.

GluR3 and plasticity

GluR3 is highly similar to GluR2 at the C-terminal sequence. In this study, we demonstrated that GluR3 knockout mice exhibited normal LTD in the ACC. This result is in accordance with a previous report, which showed the presence of GluR3-independent mechanisms in hippocampal LTD (Meng et al., 2003). GluR3-independent LTD in the hippocampus might

be related to the previous finding, in which overexpressed homomeric GluR3 receptors reached spines but were not delivered to synapses (Shi et al., 2001). Future studies are necessary to investigate the detailed molecular and cellular mechanisms of GluR3 subunit for other forms of cingulate synaptic plasticity.

Physiological significance

ACC is suggested to contribute to cognitive functions such as learning, memory, persistent pain and fear (Hayashi et al., 2000; Frankland et al., 2004; Maviel et al., 2004; Wiltgen et al., 2004; Tang et al., 2005). Although a relationship between LTD and learning and memory remains unclear, LTD seems to be one of the cellular mechanisms and play key functions for experience-dependent plasticity. The physiological significance for the LTD in the ACC is to serve as an autoregulator for synaptic strength, reducing synaptic transmission to maintain an appropriate neuronal activity (Zhuo, 2004). Our previous results showed loss of LTD in the ACC after amputation (Wei et al., 1999), which may lead to over-excitation in the ACC neurons and thus contribute to the chronic pain or unpleasantness related to the injury. Therefore, dissecting the molecular mechanisms for LTD in the ACC will be helpful to understand the synaptic basis for functional excitatory transmission and plasticity in ACC-related functions.

In summary, we showed that GluR2 mediated LTD expression in the ACC. This is the first evidence to demonstrate that GluR2-related events after LTD induction are rapidly completed and this mechanism may be important for the formation of memory. Our results suggest that GluR2 subunit is crucial for synaptic depression in the ACC and may play critical roles under physiological and pathological conditions.

Acknowledgments

Supported by grants from the Canadian Institutes of Health Research, the EJLB-CIHR Michael Smith Chair in Neurosciences and Mental Health, and the Canada Research Chair to M. Z. L.-J.W. is supported by postdoctoral fellowships from the Canadian Institutes of Health Research and Fragile X Research Foundation of Canada. We thank Dr. Z. Jia for providing GluR2 and GluR3 knockout mice. We also thank Dr. Shanelle Ko for critical reading.

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