KAINATE RECEPTOR-MEDIATED SYNAPTIC TRANSMISSION IN THE ADULT ANTERIOR CINGULATE CORTEX

Abbreviated title: Forebrain kainate receptor-mediated synaptic transmission

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Abstract

Kainate (KA) receptors are expressed widely in the central nervous system. However, little is known about their functional characterization, molecular identity and role in synaptic transmission in the forebrain of adult mice. Patch-clamp recordings in genetically modified mice show that postsynaptic KA receptors contribute to fast synaptic transmission in pyramidal neurons in the anterior cingulate cortex (ACC), a forebrain region critical for higher-order cognitive brain functions such as memory and mental disorders. Single shock stimulation could induce small KA receptor-mediated excitatory postsynaptic currents (KA EPSCs) in the presence of picrotoxin, AP-5, and a selective AMPA receptor antagonist, GYKI 53655. KA EPSCs had a significantly slower rise time course and decay time constant compared with AMPA receptor-mediated EPSCs. High frequency repetitive stimulation significantly facilitated the KA EPSCs. Genetic deletion of the GluR6 or GluR5 subunit significantly reduced, and GluR5&6 double knockout completely abolished KA EPSCs and KA-activated currents in ACC pyramidal neurons. Our results show that KA receptors contribute to synaptic transmission in adult ACC pyramidal neurons and provide a synaptic basis for the physiology and pathology of KA receptors in ACC-related functions.
Kainate (KA) receptors are one of three subtypes of ionotropic receptors for glutamate, the major excitatory neurotransmitter in the brain. The KA receptor family is composed of five different subunits, namely GluR5, GluR6, GluR7, KA1 and KA2, which can form a variety of homomeric and heteromeric receptors (Hollmann and Heinemann 1994). With the development of KA receptor specific drugs and knockout (KO) mice, studies have defined the functional roles of KA receptors in both mediating and modulating synaptic transmission, as well as synaptic plasticity (Frerking and Nicoll 2000; Huettner 2003; Lerma 2003). The postsynaptic KA receptors that mediate synaptic responses were initially found at hippocampal mossy fiber synapses (Castillo et al. 1997; Vignes et al. 1997). Subsequently, the involvement of KA receptors in synaptic transmission was reported in many central synapses, from the spinal cord to the neocortex (Ali 2003; Bureau et al. 2000; Cossart et al. 1998; DeVries and Schwartz 1999; Eder et al. 2003; Frerking et al. 1998; Kidd and Isaac 1999; Li et al. 1999).

The anterior cingulate cortex (ACC) forms a large region around the rostrum of the corpus callosum, which receives projections from the amygdala (Vogt and Pandya 1987), thalamus (Vogt et al. 1979), and hippocampus (Tamminga et al. 2000; Vogt et al. 1979). The ACC is involved in emotional and attentive responses to internal and external stimulation, such as intelligent behaviors, emotions, pain and memory (Allman et al. 2001; Derbyshire et al. 2002; Paus 2001; Zhuo 2004). Excitatory synaptic transmission within the ACC is mediated by glutamate and its receptors (Sah and Nicoll 1991; Tanaka and North 1994; Wei et al. 1999). Results from in situ hybridization and immunostaining show that KA receptor subunits are expressed in the ACC (Bahn et al. 1994; Huntley et al. 1993). For example GluR5, GluR6, GluR7 and KA2 are highly expressed, whereas KA1 is either weakly detectable during postnatal days or not expressed at all (Bahn et al. 1994). Studies have demonstrated the involvement of
KA receptors during synaptic transmission in cortical synapses in young rats (Ali 2003; Eder et al. 2003; Kidd and Isaac 1999). Despite these advances, the synaptic roles of KA receptors in the ACC of adult animals, as well as their molecular identity, have not yet to be directly addressed. In this study, we demonstrate the presence of both functional KA receptors in ACC pyramidal neurons of adult mice. Genetically modified mice with deletions of GluR5 and/or GluR6 were used to show that both GluR5 and GluR6 are involved in synaptic transmission in the adult ACC.

Materials and Methods

Animals

Adult C57BL/6 mice were purchased from Charles River (8-12 weeks old). GluR5 and GluR6 KO mice were obtained as gifts from Stephen F. Heinemann (Salk Institute, San Diego, CA) (Mulle et al. 1998; Sailer et al. 1999). For experiments using knockout mice, GluR5, GluR6, and GluR5&6 KO were maintained on a mixed 129Sv x C57BL/6 background and wild-type littermates were used as controls. The GluR5&6 KO mice were generated by crossing homozygous GluR5 and GluR6 KO mice. All mice were maintained on a 12 h light/dark cycle with food and water provided ad libitum. The Animal Care and Use Committee at the University of Toronto approved the experimental protocols. All experiments related to mutant mice were performed blind to the genotype.

Whole-cell patch clamp recordings in adult ACC slices

Adult wild-type and KO mice (8-14 weeks old) were anesthetized with 1-2 % halothane. Transverse slices of the ACC (300 μM) were prepared using standard methods (Wei and Zhuo 2001). Slices were transferred to a room temperature-submerged recovery chamber with an
oxygenated (95 % O₂ and 5% CO₂) solution containing (in mM): NaCl, 124; NaHCO₃, 25; KCl, 2.5; KH₂PO₄, 1; CaCl₂, 2; MgSO₄, 2; glucose, 10. After a 1-hour recovery period, slices were transferred into a recording chamber on the stage of an Axioskop 2FS microscope (Zeiss) equipped with infrared DIC optics for visualizing whole-cell patch clamp recordings. Excitatory postsynaptic currents (EPSCs) were recorded from layer II/III pyramidal neurons with an Axon 200B amplifier (Axon Instruments, CA) in the ACC and stimulation was delivered by a bipolar tungsten-stimulating electrode placed in layer V of the ACC (Fig. 1A). Control test pulses were given every 30 sec. For frequency facilitation, repetitive stimulation was delivered at 200 Hz (5, 10 or 20 shocks) or 25 Hz (5 shocks). In the voltage-clamp configuration, recording electrodes (2-5 MΩ) contained the pipette solution composed of (in mM): Cs-gluconate, 120; NaCl, 5; MgCl₂ 1; EGTA, 0.5; Mg-ATP, 2; Na₃GTP, 0.1; HEPES, 10; QX-314, 2; pH 7.2; 280-300 mOsmol. Access resistance was 15-35 MΩ and was monitored throughout the experiment. Data were discarded if the access changed more than 10 % during an experiment. The membrane potential was held at -65 mV throughout the experiment.

Chemicals and drug application

All chemicals and drugs were obtained from Sigma (St. Louis, MO), except for (+)-4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine (SYM 2206), (RS)-2-amino-3-(3-hydroxy-5-tert-butylisoazol-4-yl) propanoic acid (ATPA), and lidocaine N-methyl bromide quaternary salt (QX-314) which were from Tocris Cookson (Ellisville, MO). GYKI 53655 is a kind gift from Drs. John F. MacDonald and Geoffrey T. Swanson. All experiments were conducted in the presence of picrotoxin (100 µM) and DL-2-amino-5-phosphono-pentanoic acid (AP-5) (50 µM). Drugs were applied to the perfusion solution. In some experiments (Fig. 7A), a picopump (WPI pneumatic picopump, Sarasota, FL)
was used to puff-apply KA. Before establishing whole-cell recording, the drug application pipette was moved beside the neuron using a micromanipulator (Sutter MP-285, Novato, CA). The tip of the pipette was about 5-10 µm away from the neuron recorded. The diameter of the drug application pipette tip was about 3-4 µm. The pressure and duration of the puff was 15 psi and 100 ms, respectively.

**Data analysis**

Data is presented as mean ± SEM. Statistical comparisons between two groups were performed using two-tail paired or unpaired t-test, to identify significant differences. A two-way analysis of variance (ANOVA) with the post-hoc Student-Newmann-Keuls test was used for comparing more than two groups with two different factors. In all cases, $P < 0.05$ was considered statistically significant. Time constants for EPSCs were obtained by fitting one exponential function to the falling phase of the currents.

**Results**

**KA receptor mediated synaptic transmission in adult ACC neurons**

Whole-cell patch clamp recordings were performed from visually identified pyramidal cells in layers II/III of adult ACC slices. Firing properties were examined to further confirm the neuron type. Injection of depolarizing currents into most of the neurons induced repetitive action potentials with a frequency adaptation that is typical of the firing pattern of pyramidal neurons ($n = 74$ neurons). Interneuron-like fast-spiking firing patterns were also observed in a minority of neurons recorded ($n = 4$). Next, we examined if KA receptors contribute to synaptic responses in ACC neurons of adult mice when induced by local electrical stimulation (Fig. 1A). An inward EPSC was recorded in the presence of picrotoxin (100 µM) and the selective NMDA receptor
antagonist AP-5 (50 µM) with a single pulse stimulation of the ACC. To detect KA EPSCs, the selective and potent AMPA receptor antagonist, GYKI 53655 (100 µM) was then applied through the bath solution. As shown in Fig. 1B and C, GYKI 53655 had a fairly rapid and rigorous inhibitory effect on EPSCs. A small residual EPSC persisted in the presence of GYKI 53655 10 min after perfusion (-138.2 ± 17.2 pA before and -11.3 ± 1.5 pA after, n = 11 neurons/8 mice). Perfusion of the AMPA/KA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 µM) entirely blocked the residual GYKI 53655-resistant current (n = 5, Fig. 1A-C), suggesting that the current was mediated by KA receptors. To evaluate the contribution of KA EPSCs to non-NMDA receptor-mediated EPSCs, we calculated the relative peak amplitude of the GYKI 53655-resistant EPSC to the control EPSC. As calculated, the contribution of KA receptor-mediated synaptic responses was 8.9 ± 1.0 % (n = 11, Fig. 1D). These results show that a relatively small component of fast excitatory synaptic transmission is mediated by synaptic KA receptors in the adult ACC. Another AMPA receptor antagonist, SYM2206 (100 µM) was also used to dissect the KA EPSC. Residual currents were observed 10 min after SYM 2206 perfusion (-77.8 ± 1.4 pA before and -14.1 ± 0.3 pA after, n = 23/18 mice) (data not shown) and the contribution of the residual current to the control current was 18.5 ± 0.2 % (n = 23, Fig. 1D). The discrepancy between the results obtained from GYKI 53655 and SYM 2206 may result from differences in the selectivity of the compounds. Therefore, to exclude the possibility that AMPA receptors are not completely blocked by SYM2206, the following experiments studying functional KA receptors were performed in the presence of GYKI 53655 instead of SYM 2206.

**Slow kinetics of KA receptor-mediated EPSCs**
Despite the rapid desensitization and deactivation of heterologously-expressed KA receptors (Paternain et al. 1998; Swanson and Heinemann 1998), most studies report that KA receptor-mediated EPSCs have slow kinetics (Ali 2003; Bureau et al. 2000; Cossart et al. 1998; DeVries and Schwartz 1999; Frerking et al. 1998; Kidd and Isaac 1999; Li et al. 1999). We performed a quantitative study of the kinetics of KA EPSCs in adult ACC slices. As shown in Fig. 2, KA EPSCs displayed slower kinetics than AMPA EPSCs. The decay time constant of KA EPSCs were much slower than that for AMPA EPSCs in the same neurons (101.2 ± 2.3 ms vs 12.0 ± 0.7 ms, n = 11, \( P < 0.001 \)) (Fig. 2C and D). In addition, the rise time (10-90%) of KA EPSCs was also slower than the AMPA EPSCs (8.1 ± 0.4 ms vs 3.5 ± 0.3 ms, n = 11, \( P < 0.01 \)) (Fig. 2C and D). The kinetics of KA EPSCs in ACC pyramidal neurons were similar to those reported in most preparations, for example, hippocampal CA3 neurons (decay time, 103 ± 7 ms; rise time, 6.8 ± 0.3 ms) (Castillo et al. 1997) and thalamocortical neurons (decay time, 154.9 ± 25.5 ms) (Kidd and Isaac 1999).

**Summation properties of KA receptor-mediated EPSCs during repetitive stimulation**

In most synapses, brief repetitive impulse trains greatly facilitate KA receptor-mediated EPSCs (Castillo et al. 1997; Mulle et al. 1998; Vignes et al. 1997). To determine the summation properties of KA receptor-mediated synaptic responses in the ACC, repetitive stimulation was applied. As shown in Fig. 3A, in the presence of GYKI 53655, a small residual KA EPSC was significantly increased in amplitude after repetitive stimulation (200 Hz, 5 shocks, from -8.9 ± 1.1 pA to -19.7 ± 2.4 pA, n=8, \( P < 0.001 \), see Fig. 4). The increased current could largely be blocked by CNQX, indicating that the current was mediated by KA receptors. To examine whether the summation of KA EPSCs is due to the slow kinetics of the current, comparatively
low frequency repetitive stimuli (25 Hz, 5 shocks) were also applied. As shown in Fig. 3B, significant summation of KA EPSCs, but not AMPA EPSCs, was observed (n=5).

The amplitude of KA EPSCs was dependent on the number of stimuli delivered (Fig. 4). Five shocks of 200 Hz train significantly increased single shock induced KA EPSCs. A further increase could be induced by 10 shocks (n=8, $P < 0.01$), but no additional increase was observed by 20 shocks when compared to those obtained by 10 shocks, suggesting the saturation of the KA EPSCs (Fig. 4A and B). The enhancement of KA EPSCs was not associated with a change in the decay time of the current. When fitted with a single-exponential function, no difference was found in the decay time constant for the currents induced by different numbers of stimulation (Fig. 4B).

**Voltage-dependence of KA receptor-mediated EPSCs**

To further characterize synaptic KA receptors, we studied the current-voltage (I-V) relationship of the KA EPSCs. The I-V relationship of KA receptors can reflect the calcium permeability and the subunit composition of channels (Egebjerg and Heinemann 1993; Ruano et al. 1995). In the presence of GYKI 53655, KA EPSCs were induced by a single shock. When recorded at various holding potentials ranging from -70 mV to +50 mV, KA EPSCs reversed at a potential of ~ 0 mV ($5.2 \pm 3.3$ mV, n = 6 neurons/4 mice, Fig. 5A). The current recorded at the peak amplitude in relation to the holding potential was then plotted. Fig. 5B illustrates the I-V curve of KA EPSCs in adult ACC neurons, which displays a strong outward rectification (Fig. 5B). The mean rectification index of the KA EPSCs (ratio of estimated conductance at +40 mV and -60 mV) was $4.9 \pm 0.8$ (n=6).

**KA receptor-mediated EPSCs in wild-type, GluR5 KO and/or GluR6 KO mice**
Next, we wanted to study the subunit composition of synaptic KA receptors in adult ACC neurons using mutant mice lacking GluR5, GluR6, or GluR5&6 subunits. Since the proportion of KA EPSCs to control EPSCs was small in wild-type mice (8.9 ± 1.0 %, Fig. 1), evaluation of their contribution in knockout mice is difficult. Therefore, we employed two methods to evaluate the contribution of GluR5 and GluR6 in mediating the postsynaptic currents. First, we compared KA receptor-mediated currents induced by high frequency stimulation (200 Hz, 10 shocks) in wild-type and knockout mice. In the presence of picrotoxin, AP-5 and GYKI 53655, high frequency stimulation induced large KA receptor-mediated currents in slices of wild-type mice (Fig. 4B). As shown in Fig. 6A and C, KA EPSCs induced by repetitive stimulation in GluR5 KO mice were significantly reduced compared to that of wild-type mice (wild-type, -23.1 ± 2.5 pA, n = 8 neurons/6 mice; GluR5 KO, -16.2 ± 1.8 pA, n = 7 neurons/6 mice; P < 0.05). In GluR6 KO mice, a dramatic decrease in KA receptor-mediated currents was also observed (GluR6 KO, -8.5 ± 2.6 pA, n = 8 neurons/6 mice; P < 0.01). There was a significant difference in KA EPSCs between GluR5 KO and GluR6 KO mice (P < 0.05), suggesting that GluR6 plays a more important role in KA EPSCs. In GluR5&6 KO mice, repetitive stimulation induced almost no current (-1.6 ± 0.2 pA, n = 5 neurons/4 mice). These results suggest that both GluR5 and GluR6 are involved in KA EPSCs but GluR6 is the major contributor. To further confirm the results, we compared the input (stimulation intensity)-output (KA EPSC amplitude) relationship of KA EPSCs in wild-type and knockout mice. As shown in Fig. 6B and D, both GluR5 and GluR6 KO mice showed a significant decrease in KA EPSCs. Double KO mice showed no current under different intensities of stimulation. Taken together, these results indicate that both GluR5 and GluR6 underlie the synaptic KA receptor-mediated current in ACC pyramidal neurons.
**Kainate-activated currents in wild-type, GluR5 KO and/or GluR6 KO mice**

To further study the subunit composition of functional KA receptors in adult ACC pyramidal neurons, KA receptor agonists were applied to evoke whole-cell currents in wild-type and KO mice. First, KA was puff-applied (15 psi, 100 ms) in the presence of picrotoxin, AP-5 and GYKI 53655 to neurons in ACC slices. As shown in Fig. 7A and B, 10 µM KA activated currents were slightly reduced in GluR5 KO mice (wild-type, -24.5 ± 1.9 pA, n=7 neurons/4 mice; GluR5 KO, -19.0 ± 2.2 pA, n=6 neurons/4 mice, \( P < 0.05 \)). However, a greater reduction of KA-activated current was found in GluR6 KO mice (- 3.3 ± 0.2 pA, n=6 neurons/4 mice, \( P < 0.001 \)) as compared with that in GluR5 KO mice. No current was observed in double KO mice when 10 µM of KA was puff-applied (n=5 neurons/3 mice). In addition, the selective agonist for GluR5, ATPA, was used to directly assess the contribution of GluR5 in functional KA receptors in adult ACC pyramidal neurons. As shown in Fig. 7C and D, 10 µM of ATPA induced small currents in both wild-type (-11.5 ± 1.0 pA, n = 4 neurons/3 mice) and GluR6 KO mice (-14.2 ± 1.2 pA, n = 4 neurons/3 mice). However, little or no detectable current was observed in GluR5 KO (n = 4) and GluR5&6 KO mice (n = 4). Taken together, these results suggest that both GluR5 and GluR6 are involved in functional KA receptors but GluR6 plays a primary role in ACC pyramidal neurons.

**Discussion**

Our present results provide electrophysiological and genetic evidence that KA subtype receptors contribute to fast excitatory synaptic transmission in the ACC of adult mice. Based on their firing properties, as well as morphological identification using Lucifer yellow (unpublished data), the recorded neurons were mostly cingulate pyramidal neurons. Using whole-cell patch
clamp recordings in neurons of ACC slices, functional KA receptors were studied by stimulation-evoked EPSCs and agonist-activated currents, respectively. Considering the cumulative physiological evidence for the role of the ACC in long-term memory, persistent pain and higher brain functions, the present study provides important synaptic mechanisms for signal transmission in the forebrain. Second, due to the lack of selective KA receptor subunit antagonists, it is difficult to investigate the contribution of KA receptor subunits, such as GluR5 and GluR6 to central synaptic transmission. Using GluR5 and GluR6 KO mice, we provide the first evidence, to our knowledge, for kainate receptor-mediated synaptic transmission in the cingulate cortex. Our results indicate that both GluR5 and GluR6 contribute to functional KA receptors. Although the stimulating electrode did not selectively activate a specific population of synapses in the ACC (e.g., local excitatory synapses versus projecting synapses from other central nuclei), we believe our results provide a foundation for future experiments to dissect the KA receptor subunit contribution in cingulate circuits.

We initially used SYM 2206 to separate KA EPSCs from AMPA EPSCs. SYM 2206 was reported to be a selective AMPA receptor antagonist (Pelletier et al. 1996). Previous studies have successfully isolated KA EPSCs in spinal dorsal horn neurons using this compound (Li et al. 1999). In addition, several other groups have employed SYM 2206 to block AMPA receptors (Binns et al. 2003; Wilding and Huettner 2001). The concentration (100 µM) of SYM 2206 that was used could produce maximal inhibition of AMPA receptors (half-maximal inhibitory concentration= 1-2 µM) but less than 20-30% inhibition of kainate receptors (Li et al. 1999; Pelletier et al. 1996). To confirm the KA EPSCs, we used another selective AMPA receptor antagonist, GYKI 53655 (Paternain et al. 1995; Wilding and Huettner 1995). Our results showed a larger residual current after application of SYM 2206 (18.5 ± 0.2 %) than after GYKI 53655.
(8.9 ± 1.0 %) (Fig. 1). Moreover, we found that the residual current in the presence of SYM2206 had faster kinetics than those in the presence of GYKI 53655 and there still remained a small residual synaptic current in GluR5&6 KO mice in the presence of SYM 2206 (data not shown). We therefore speculate that SYM 2206 may incomplete block AMPA receptor mediated EPSCs. Thus, it should be prudent to use SYM2206, when unmasking KA-mediated EPSCs.

The contribution of KA EPSCs is very small (8.9 ± 1.0 %) in ACC pyramidal neurons (Fig. 1). Moreover, saturated KA receptor EPSCs are only about 20 pA after high frequency repetitive stimulation (Fig. 3). A small percentage of KA EPSCs (< 10%) compared with AMPA EPSCs is reported in most preparations; for example, Golgi cells in the cerebellum (Bureau et al. 2000) and layer V pyramidal neurons in the neocortex (Eder et al. 2003). However, a comparatively bigger component of KA receptor-mediated EPSCs was present in thalamocortical synapses (Kidd and Isaac 1999), spinal dorsal neurons (Li et al. 1999) and the basolateral amygdala (Li and Rogawski 1998). The small size of the current may be due to the small channel conductance of KA receptors (Swanson et al. 1996) in ACC pyramidal neurons. Moreover, the contribution of KA receptor mediated currents is age-dependent (Kidd and Isaac 1999). The use of adult mice in the present study may partially explain why the currents were so small. The strong outward rectification of the I-V curve for KA EPSC was observed in adult ACC neurons (Fig. 5). Similar results were reported in pyramidal neurons in layer II/III and layer V in motor cortex (Ali 2003). These results suggest that edited KA receptors are present in adult ACC neurons since the edited form of GluR6 exhibits an outward rectification and less Ca\(^{2+}\) permeability compared to unedited GluR5 and/or GluR6 subunits which are significantly permeable to calcium ions and show a strong inwardly rectified I-V curve (Egebjerg and Heinemann 1993; Ruano et al. 1995).
Although heterologously expressed kainate receptors show fast kinetics similar to AMPA receptors (Paternain et al. 1998; Swanson and Heinemann 1998), KA EPSCs exhibited slower kinetics compared with AMPA EPSCs recorded in the same cell (Ali 2003; Bureau et al. 2000; Cossart et al. 1998; DeVries and Schwartz 1999; Frerking et al. 1998; Kidd and Isaac 1999; Li et al. 1999). It is now generally accepted that the kinetics of KA EPSCs might be due to intrinsic properties of these postsynaptic receptors rather than their extrasynaptic location (Lerma 2003). For example, interactions of KA receptors with intracellular proteins have been reported to affect kainate receptor kinetics (Bowie et al. 2003; Garcia et al. 1998). In adult ACC slices, we also observed slow kinetics of KA EPSCs. Both the rise time and decay time constant of GYKI 53655-resistant KA EPSCs were significantly slower than that of control currents (Fig. 2). Moreover, the decay time of the current was not associated with an increase in the number of stimuli (Fig. 3B). Therefore, the slow kinetics of KA receptor EPSCs in the ACC may be due to the receptor itself rather than spill over of synaptically released glutamate. Recently, however, it is interesting to note that fast kinetics of KA receptor EPSCs have been reported in a minority of synapses (Ali 2003; DeVries and Schwartz 1999; Eder et al. 2003).

Our results show that the knockout of GluR6 had a greater affect on both KA EPSCs and KA-induced currents compared to the deletion of GluR5. Functional GluR6 receptors have been reported in most pyramidal neurons and some interneurons in different nuclei, including hippocampal CA3 and CA1 neurons (Bureau et al. 1999; Mulle et al. 1998; Mulle et al. 2000), striatum and nucleus accumbens neurons (Casassus and Mulle 2002; Chergui et al. 2000), Golgi cells (Bureau et al. 2000) and spinal dorsal horn neurons (Kerchner et al. 2002). Deletion of GluR5 also reduced synaptic KA receptor-mediated EPSCs and the current evoked by KA perfusion, although the reduction was smaller when compared to GluR6 KO mice. As expected,
the GluR5 selective agonist, ATPA, induced small inward currents in both wild-type and GluR6 KO mice. In GluR5&6 KO mice, both synaptic KA receptor EPSCs and KA-activated currents were completely abolished. Taken together, these results clearly highlight an important role for the GluR6 subunit, and a less prominent role for GluR5, in functional KA receptors in ACC pyramidal neurons. Recently, KO mice were used to show the involvement of the GluR6, but not GluR5, kainate receptor subunits in synaptic plasticity within the amygdala and auditory cortex (Ko et al. 2005a). However, it is generally difficult to assess the exact contribution of GluR5 and GluR6 in native KA EPSCs, considering GluR5 can co-assemble with GluR6 to form functional KA receptors (Kerchner et al. 2002; Mulle et al. 2000; Paternain et al. 2000) and functional compensation likely takes place in KO mice (Christensen et al. 2004). With the exception of KA1, the other four KA receptor subunits were shown to be expressed in the ACC (Bahn et al. 1994; Huntley et al. 1993). Therefore, it is possible that subunits other than GluR5 and GluR6, for example GluR7 and KA2, are also involved in functional KA receptors within the ACC. However, in GluR5&6 KO mice, neither KA EPSCs nor KA-activated currents were observed. Thus, whether KA2 and Glu7 subunits may co-assemble with GluR5 and GluR6 to form native kainate receptors in the ACC remains unclear. In order to dissect the exact composition of KA receptors in the ACC selective KO mice for the other KA receptor subunits, as well as selective pharmacological tools, are needed.

Recent studies from both humans and animals suggest that the ACC, and its related areas, are important for the processing of sensory information, learning and memory, emotion and other higher-order brain functions (Calejesan et al. 2000; Casey et al. 1996; Davis et al. 2000; Davis et al. 1997; Devinsky et al. 1995; Donahue et al. 2001; Eisenberger et al. 2003; Johansen et al. 2001; Koyama et al. 1998; Kwan et al. 2000; Wei and Zhuo 2001). Our previous results show
that the ACC is involved in pain and fear memory in rats or mice (Calejesan et al. 2000; Ko et al. 2005b; Tang et al. 2005; Wei and Zhuo 2001). Thus, understanding synaptic mechanisms within the ACC will give us insight into plastic changes related to pain, memory and mental diseases. The present study demonstrates that glutamate KA receptors are located in ACC synapses and provides a synaptic basis for the physiology and pathology of KA receptors in ACC-related functions.
Figure legends

**Fig. 1. KA receptor-mediated EPSCs in adult ACC pyramidal neurons**

A. Diagram showing the placement of stimulating and recording electrodes in the ACC.

B. Control EPSCs were recorded in the presence of picrotoxin (PTX, 100 µM) and AP-5 (50 µM). After the perfusion of GYKI 53655 (100 µM), a small residual current remained, which could be totally blocked by CNQX (20 µM). In the following example of EPSCs, each trace represents an average of 5-10 consecutive recordings.

C. Sample points showing the time course of GYKI 53655 and CNQX effects on the neuron shown in B.

D. Statistical results showing the percentage of EPSCs in the presence of SYM 2206, GYKI 53655 or CNQX.

**Fig. 2. KA receptor-mediated EPSCs show slower kinetics**

A. Superimposed traces showing control EPSCs, EPSCs after application of GYKI 53655 or CNQX.

A. Enlarged traces showing the traces in A. Peak current of control EPSCs is off-scale to emphasize the small GYKI-resistant EPSC.

E. Scaled traces showing the different kinetics for GYKI-sensitive and GYKI-resistant current.

F. Statistical results for the rise time and decay time constant of GYKI-sensitive and GYKI-resistant current.

**Fig. 3. Summation of KA receptor-mediated EPSCs by repetitive stimulation**

A. Repetitive stimulation (200 Hz, 5 shocks) increased KA EPSCs and CNQX could block the current.
B. Repetitive stimulation (25 Hz, 5 shocks) induced summation of KA EPSCs but not AMPA EPSCs. AMPA EPSCs were obtained by subtraction of KA EPSCs and KA EPSCs were obtained by subtraction of the artifact in the presence of CNQX.

**Fig. 4. Intensity-dependent summation of KA receptor-mediated EPSCs**

A. Representative traces of KA EPSCs obtained after different number of stimuli at a frequency of 200 Hz.

B. Statistical results show peak amplitude of the KA EPSC by different stimuli (200 Hz) versus numbers of stimulation (200 Hz). Note that 10 shocks induced a saturated current.

**Fig. 4. Current-voltage relationship for KA receptor-mediated EPSCs**

A. KA receptor-mediated EPSCs were recorded at holding potentials from -70 mV to 50 mV.

B. Current-voltage plot for KA EPSCs. The currents exhibit strong outward rectification and the reversal potential is $5.2 \pm 3.3$ mV $(n = 6)$.

**Fig. 5. Comparison of KA receptor-mediated EPSCs in wild-type, GluR5 KO and/or GluR6 KO mice**

A and C. Repetitive stimulation (200 Hz, 10 shocks) induced KA receptor EPSCs in wild-type, GluR5, GluR6 and GluR5&6 KO mice. Amplitude of KA receptor-mediated EPSCs was significantly decreased in GluR6, GluR5, and GluR5&6 KO mice. In the present and following figure, * denotes significant difference between KO and wild-type mice; † denotes significant difference between GluR5 and GluR6 KO mice; # denotes significant difference between GluR5&6 KO and GluR6 KO mice.

B and D. Input-output relationship for single shock induced KA receptor EPSCs in wild-type, GluR5, GluR6 and GluR5&6 KO mice, revealing the contribution of both GluR5 and GluR6 in KA receptor EPSCs in the ACC.
Fig. 6. Comparison of the responses of ACC neurons to kainate and ATPA in wild-type and GluR5 and/or GluR6 KO mice

A and B. Sample traces and statistical results showing whole-cell currents activated by puff-application of 10 μM KA in the presence of PTX, AP-5 and GYKI 53655 in wild-type and KO mice. The responses were dramatically decreased in GluR6 KO, while only mild decreases were observed in GluR5 KO mice.

C and D. Sample traces and statistical results showing whole-cell currents activated by perfusion of 10 μM ATPA in wild-type and KO mice. Note that no detectable current was observed in GluR5 KO and GluR5&6 KO mice.
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Figure 1
Figure 2
Figure 3

**A**
- GYKI 53655
- 200 Hz, 5 shocks
- GYKI 53655
- CNQX

**B**
- 25 Hz, 5 shocks
- CNQX
- GYKI-sensitivity
- GYKI-resistant

Control
Figure 4
Figure 5
Figure 7