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ORIGINAL ARTICLE

Cortical GluK1 kainate receptors modulate scratching in adult mice

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Abstract

Recent investigations into the mechanisms mediating itch transmission have focused on spinal mechanisms, whereas few studies have investigated the role of the cerebral cortex in itch-related behaviors. Human imaging studies show that several cortical regions are active in correspondence with itch, including the anterior cingulate cortex (ACC). We present here evidence of cortical modulation of pruritogen-induced scratching behavior. We combine pharmacological, genetic, and electrophysiological approaches to show that cortical GluK1-containing kainate (KA) receptors are involved in scratching induced by histamine and non-histamine-dependent itching stimuli. We further show that scratching corresponds with enhanced excitatory transmission in the ACC through KA receptor modulation of inhibitory circuitry. In addition, we found that inhibiting GluK1-containing KA receptors in the ACC also reduced behavioral nociceptive responses induced by formalin. Our results reveal a new role of the cortex in pruritogen-induced scratching.

Keywords: cortex, glutamatergic transmission, inhibitory transmission, itch, kainate receptors, scratching.

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Itch is an uncomfortable sensation that strongly evokes a desire to scratch the area involved. Itch can be induced through histamine dependent (Ikoma *et al.* 2006; Davidson and Giesler 2010) or independent mechanisms (Steinhoff *et al.* 2003; Ikoma *et al.* 2006). Both types of itch have been found to activate spinothalamic tract neurons (Davidson *et al.* 2007; Namer *et al.* 2008), and human imaging studies show they correspond with activity in the anterior cingulate cortex (ACC) (Ikoma *et al.* 2006; Yosipovitch *et al.* 2008; Papoiu *et al.* 2012). The ACC receives projections from the thalamus and is involved in emotional and attentive responses to noxious stimulation (Rainville *et al.* 1997; Lee *et al.* 2007; Zhuo 2008).

Glutamate may be a key neurotransmitter mediating itch- and pain-induced behaviors. For example, disinhibition of dorsal horn neurons results in exaggerated scratching behavior in transgenic Bhlhb5 null mice (Ross *et al.* 2010), indicating that scratching can be modulated by central circuits. Similarly, glutamate is a key neurotransmitter for gastrin-releasing peptide (GRP)-dependent and independent synaptic transmission in rat dorsal horn neurons (Koga *et al.* 2011), and spinal horn GRP receptors are involved in scratching (Sun and Chen 2007; Sun *et al.* 2009). Importantly, spinal dorsal horn neurons are activated by pruritogens and noxious stimuli (Ikoma *et al.* 2006; Davidson *et al.* 2007), and glutamate is the major fast excitatory neurotransmitter for transmission in the dorsal horn (Yoshimura and Jessell 1989; Li *et al.* 1999; Koga *et al.* 2011) which can be modulated by cortical activity (Calejesan *et al.* 2000). Whether scratching can be modulated via cortical mechanisms however remains to be addressed. Moreover, despite mounting neuroimaging data implicating cortical activity in itch and pain (Rainville *et al.* 1997; Drzezga *et al.* 2001;

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Abbreviations used: ACC, anterior cingulate cortex; AMPA, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid; EPSCs, excitatory post-synaptic currents; EPSPs, excitatory post-synaptic potentials; GRP, gastrin-releasing peptide; PAR2, protease-activated receptor-2; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

Ikoma *et al.* 2006; Leknes *et al.* 2007; Yosipovitch *et al.* 2008; Kleyn *et al.* 2012; Papoiu *et al.* 2012), no study has investigated whether they involve similar cortical mechanisms.

In the ACC, kainate (KA) receptor GluK1 subunits mediate small excitatory post-synaptic currents (EPSCs) (Wu *et al.* 2005), which increase in frequency and amplitude in response to high frequency stimulation. Most investigations of ACC excitatory transmission have focused on glutamate mediated 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) and *N*-Methyl-D-aspartate (NMDA) receptor function (Zhuo 2008), whereas little is known regarding the role of cortical KA receptors. Behavioral observations of GluK1$^{-/-}$ mice indicate that they modulate behavioral responses to noxious and emotional stimuli (Ko *et al.* 2005). To our knowledge, the possible contributions of ACC KA receptors to behavioral responses to itch or pain have not been reported. Here, we combined behavioral, pharmacological, electrophysiological, and genetic approaches to investigate the role of ACC KA receptors in pruritogen-induced scratching, and show for the first time that GluK1-containing KA receptors within the ACC are involved in scratching behavior, and provide evidence of cortical modulation of behavioral responses to itch- and inflammatory pain-inducing stimuli.

Materials and methods

Animals

C57BL/6 mice (Charles River, Quebec, QC, Canada) were used for most experiments. Preliminary behavior data were collected using GluK1$^{-/-}$ mice (gift from Dr. Stephen F. Heinemann; The Salk Institute, San Diego) which were maintained on a C57BL/6 background; previous observations showed no differences in scratching between adult C57BL/6 and wild-type littermates. Transgenic FosGFP mice were a gift from the laboratory of Dr. Alison Barth (Carnegie Mellon University). All experiments were performed with adult (8–12 weeks) male mice. Animals were housed under a 12 h light/dark cycle (lights on at 7:00 A.M.), and had access to food and water *ad libitum*. All experiments were performed according to the recommendations of the Canadian Council on Animal Care, and were approved by the University of Toronto Animal Care Committee.

Behavioral scratching

Twenty-four hours prior to the experiment, the fur on the nape of the neck was shaved so that an area of exposed skin (~ 0.5 cm × 0.5 cm) was clearly visible. On the day of testing, mice were placed in empty home cage containers, and allowed to acclimatize for 30 min prior to pruritogen application. For some experiments, saline or UBP-302 was administered 30 min (i.p. injections) or 15 min (ACC microinjections) prior to pruritogen application. Scratching behavior was quantified for 30-min post-application of pruritogens, where we defined one bout of scratching by either hind paw directed toward the nape of the neck as a scratching episode. Behavioral observations were performed blind.

Behavioral acute nociception

In the hotplate test, we placed mice on a standard heated (55°C) thermal hotplate (Columbus Instruments, Columbus, OH, USA). Nociceptive response latencies were recorded six times and averaged, with 10 min between trials. We also assessed latencies of the spinal nociceptive tail-flick reflex by exposing the underside of the tail of mice to radiant heat (Columbus Instruments). All tests were performed blind.

Rota-Rod

Motor functions in C57 mice exposed to saline or UBP-302 (i.p. 50 mg/kg) were tested through the Rota-Rod test (Med Associates, St Albans, VT, USA) as described previously (Wu *et al.* 2007). Briefly, mice were trained on a rotating rod at a constant velocity of 16 rpm until they could remain on for a 30 s period; 24 h later, mice were either exposed to systemic injections of saline or UBP-302, and were tested on the same apparatus but with the velocity increasing from 2.14×10^{-4} to 2.14×10^{-2} *g* over a 5 min period. Measures were taken of the duration each animal was able to maintain its balance walking on the rotating drum. Mice were given three trials with a maximum time of 300 s and a 5 min inter-trial rest interval. The latency to fall was taken as a measure of motor function. If a mouse gripped on to the rotating drum and remained on without walking, a fall was recorded after two complete rotations. All tests were performed blind.

Itch-inducing stimuli (pruritogens)

Histamine (250 or 18 μg/10 μL, dissolved in saline; Sigma-Aldrich, Oakville, ON, Canada), protease-activated receptor-2 (PAR2) agonist peptide, Ser-Leu-lle-Gly-Arg-Leu-NH2, (SLIGRL-NH_2) (25 μg/10 μL, dissolved in saline; Tocris, Bristol, UK), or saline (10 μL) was injected intradermally into the nape of the neck via a 30-gauge Hamilton syringe. Cowhage (Mucuna pruriens) spicules were applied to the exposed skin on the nape of the neck. Cowhage pods were briefly rubbed onto the skin, until visible spicules were seen covering the exposed area.

Drugs

UBP-302 (Tocris) was dissolved in dimethylsulfoxide (DMSO) to a concentration of 50 mM. Further dilutions were made with sterile saline. For ACC microinjections, concentrations of 20 nM, 2 μM, 200 μM, and 3 mM were used, and for i.p. injections 50 mg/kg. Cortical vehicle (6% DMSO in sterile saline) injections were used as control. D-AP-5 (Tocris) CNQX sodium (Tocris) were dissolved in sterile saline and microinjected into the ACC at concentrations of 10 mM and 1 mM, respectively; sterile saline was used as control.

ACC cannulae implantation and microinjection

We implanted bi-lateral cannulas into the ACC of mice as reported previously (Li *et al.* 2010; Descalzi *et al.* 2012). Briefly, mice were anaesthetized with inhaled 2–3% isoflurane, and their heads secured on a stereotaxic frame, where the fur above the skull was shave, disinfected, and cut to expose the skull. Small holes were drilled 0.7 mm anterior to bregma and ± 0.3 mm lateral from the midline, followed by bilateral implantation of 24-gauge guide cannulas 0.9 mm beneath the surface of the skull. Two weeks after surgery, bi-lateral intra-ACC injections were delivered via a 30-gauge injection cannula which was lowered 0.85 mm deeper into the

brain than the implanted cannula guide. All intra-ACC injections consisted of 0.5 μL of solution delivered at a rate of 0.05 μL/min. Accuracy of injection sites were confirmed at the end of all experiments, data collected from injections outside of the ACC region were excluded from the study.

Immunohistochemistry

Immunostaining was performed as described previously (Li *et al.* 2010; Descalzi *et al.* 2012). Briefly, we first exposed mice to histamine or saline, and 90 min later they were heavily anesthetized with isoflurane and perfused via the ascending aorta with 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4), immediately followed by 4% paraformaldehyde (PFA) in 0.1 mol/L PB at 4°C. Brains were removed, post-fixed for 4 h in 4% PFA, and then submerged in 30% sucrose in 0.1 mol/L PB 4°C for at least 48 h. Thirty micrometer thick brain sections containing the ACC were cut using a cryostat (Leica Microsystems GmbHWetzlar, Wetzlar, Germany). Brain slices were then sequentially incubated in (i) anti- c-fos (1 : 12 000; Abcam, Toronto, ON, Canada) primary antibody for 3 days at 4°C. (ii) Biotin labeled goat anti-rabbit secondary antibody (1 : 1000; Santa Cruz, CA, USA) for 24 h at 4°C, and (iii) Cy3 conjugated streptavidin (1 : 1000; Santa Cruz, CA, USA) for 2 h at ~ 25 °C. All antibodies were diluted in PBS solutions containing 3% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) and 0.3% Triton X-100. In between each step, sections were rinsed with PBS three times for 10 min. Sections were then mounted on gelatin coated slides, air-dried, cleared and cover-slipped, and scanned using a confocal microscope (FV-1000; Olympus, Center Valley, PA, USA). All slices were scanned and analyzed blind.

Electrophysiology

Thirty minutes after intradermal injections of histamine or saline, coronal brain slices (300 μm) at the level of the ACC were prepared using standard methods (Wu *et al.* 2005; Xu *et al.* 2008; Li *et al.* 2010). Slices were then 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, and 10 glucose at ~ 25 °C for at least 1 h. Experiments were performed in a recording chamber on the stage of a BX51W1 microscope equipped with infrared differential interference contrast optics for visualization. Pyramidal neurons were identified by injecting depolarized currents into neurons to induce action potentials. Spontaneous excitatory post-synaptic currents (sEPSCs) in histamine, saline groups and Fos GFP mice were recorded under voltage clamp mode (V_H = −60 mV) (Li *et al.* 2010). Evoked EPSCs (eEPSCs) were recorded from layer II/III neurons with an Axon 200B amplifier (Molecular Devices Inc., Sunnyvale, CA, USA), and the stimulations were delivered by a bipolar tungsten stimulating electrode placed in layer V/VI of the ACC. The eEPSCs were recorded under voltage clamp mode (V_H = −60 mV) and were induced by repetitive stimulations at 0.05 Hz in the presence of picrotoxin (100 μM). KA mediated eEPSCs was isolated by GYKI 53655 (100 μM) for 10 min under voltage clamp mode (V_H = −60 mV) in the presence of picrotoxin (100 μM) and D-2-amino-5-phosphono-pentanoic acid (AP-5; 50 μM) (Wu *et al.* 2005). For frequency facilitation, repetitive stimulation was delivered at 200 Hz (5, 10, or 20 shocks) (Wu *et al.* 2005). Evoked excitatory post-synaptic potentials (EPSPs) were recorded from layer II/III and delivered in layer V/VI under current clamp mode (I = 0 pA). The recording pipettes (3–5 MΩ) were filled with a solution containing (in mM) 124 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na_3-GTP, and 10 phosphocreatine disodium (adjusted to pH 7.2 with KOH). Cells, where the initial access resistance was 15–30 MΩ, were used for experiments, and it was again tested at the end of the recordings. Data were discarded if the access resistance changed > 15%. Data were filtered at 1 kHz, and digitized at 10 kHz. All recordings were performed blind.

Data analysis

Results are expressed as mean ± SEM. Statistical comparisons were made using unpaired Student's t-tests or one-way analysis of variance (ANOVA) adjusted by the Holm–Sidak test for multiple comparisons. In all cases, $p < 0.05$ was considered statistically significant.

Results

KA receptor GluK1 subunits modulate scratching induced by different classes of itching stimuli

There is no report to date that has investigated the possible role of KA receptors in pruritogen-induced scratching. We thus first collected preliminary data using GluK1$^{-/-}$ mice backcrossed with a C57 background to investigate if GluK1 containing KA receptors may possibly play a role in pruritogen-induced behavior. We exposed adult male GluK1$^{-/-}$ mice and separately bred, age-matched C57 mice to intradermal injections of histamine (250 μg/10 μL) into the shaved nape of the neck and observed scratching behavior for 30-min post-injection; similar concentrations have been used in previous studies of itch pathways in rodents (Jinks and Carstens 2000; Sun and Chen 2007; Koga *et al.* 2011). Histamine injections caused a robust scratching response in C57 mice, which displayed on average over 170 bouts of scratching over a 30-min period. In contrast, GluK1$^{-/-}$ mice showed significantly less scratching behavior, displaying on average 60 bouts over 30 min (C57: 176 ± 18 bouts, n = 9; GluK1$^{-/-}$: 60 ± 3 bouts, n = 10, t = 6.6, $p < 0.001$; Figure S1a). We repeated these experiments with a much lower concentration of histamine (18 μg/10 μL) and once again observed significantly less scratching in GluK1$^{-/-}$ mice compared to C57 mice (C57: 63 ± 6 bouts, n = 7; GluK1$^{-/-}$: 29 ± 6 bouts, n = 7, t = 3.9, p = 0.002; Figure S1b). These results implicate GluK1-containing KA receptors in histamine-induced scratching behavior. Antihistamines however are not always effective treatments against itch, and several studies have reported histamine independent itch transmission (Davidson *et al.* 2007; Johanek *et al.* 2008; Namer *et al.* 2008; Papoiu *et al.* 2012). To determine if GluK1$^{-/-}$ mice also display altered scratching in response to non-histamine-induced itch, we

applied intradermal injections of the protease-activated receptor-2 (PAR2) agonist peptide, SLIGRL-NH_2 (25 μg/10 μL), which has been previously used by multiple studies investigating histamine-independent itch (Akiyama *et al.* 2009). Similar to our histamine experiments, GluK1$^{-/-}$ mice displayed significantly less bouts of scratching than C57 mice (C57: 74 ± 9 bouts, $n = 7$; GluK1$^{-/-}$: 36 ± 6 bouts, $n = 7$, $t = 3.6$, $p = 0.004$; Figure S1c). In addition, we also tested the effects of the naturally occurring PAR2 agonist, cowhage (*Mucuna pruriens*) (Shelley and Arthur 1955), a legume whose active component (a cysteine protease called mucunain) potently activates PAR2 receptors (Shelley and Arthur 1955; Davidson *et al.* 2007; Johanek *et al.* 2007; Reddy *et al.* 2008) and is insensitive to antihistamine treatment (Johanek *et al.* 2008). Application of cowhage spicules to the exposed nape of the neck induced obvious scratching behavior in mice, and in accordance with our histamine and SLIGRL-NH_2 observations, GluK1$^{-/-}$ mice exhibited significantly less bouts of scratching than age match C57 mice (C57: 63 ± 8 bouts, $n = 8$; GluK1$^{-/-}$: 16 ± 6 bouts, $n = 8$, $t = 4.6$, $p < 0.001$; Figure S1d). These results indicate that GluK1 receptors are involved in histamine and non-histamine-induced scratching behavior; although please note that C57 wild-types used in these preliminary experiments are inbred C57 mice while the GluK1$^{-/-}$ mutant mice were bred as a separate homozygous colony. Separately bred C57 and GluK1 WT littermates however showed similar levels of scratching in preliminary studies with compound 48/80 (Figure S2).

Pharmacological inhibition of GluK1-containing KA receptors attenuates scratching

We next sought to determine if pharmacological inhibition of KA receptor GluK1 subunits could attenuate itch-induced scratching, and exposed adult C57 mice to systemic, intraperitoneal (i.p.) injections of varying doses (1,10, or 50 mg/kg) of the selective GluK1 antagonist UBP-302 (Jane *et al.* 2009), or saline 30 min prior to histamine application. In accordance with our knockout data, we observed that UBP-302 dose-dependently attenuated histamine-induced scratching behavior. Specifically, C57 mice exposed to saline displayed significantly more scratching bouts compared to all three doses of UBP-302, and mice exposed to the larger dose of 50 mg/kg showed significantly less scratching than those exposed to lower doses of 1 and 10 mg/kg (saline: 156 ± 7 bouts, $n = 6$; UBP-302$_{(1\ mg/kg)}$: 94 ± 16 bouts, $n = 6$; UBP-302$_{(10\ mg/kg)}$: 84 ± 15 bouts, $n = 6$; UBP-302$_{(50\ mg/kg)}$: 43 ± 7 bouts, $n = 9$, $F_{3,19} = 25.5$, $p < 0.001$; Fig. 1a). Importantly, GluK1$^{-/-}$ mice exposed to i.p. injections of the largest dose of UBP-302 (50 mg/kg) and GluK1$^{-/-}$ mice exposed to saline showed similar scratching levels (saline: 55 ± 3 bouts, $n = 8$; UBP-302: 45 ± 4 bouts, $n = 8$; Fig. 1b), confirming that the reduction in scratching caused by UBP-302 is being mediated via GluK1-containing KA receptors. In addition, the RotaRod test for locomotor performance yielded similar results in C57 mice exposed to saline or UBP-302 (50 mg/kg) (Saline: 209 ± 11 s, $n = 6$; UBP-302: 199 ± 11 s, $n = 6$, $t = -5.9$, $p = 0.57$; Fig. 1c), excluding the possibility that the effects produced by UBP-302 are because of disruptions of motor ability.

ACC GluK1 KA receptors are involved in histamine and non-histamine-dependent scratching

We have presented evidence that GluK1-containing KA receptors are involved in histamine and non-histamine-induced scratching, and human imaging reports have shown that the ACC is activated by histamine, cowhage, and house allergens (Leknes *et al.* 2007; Papoiu *et al.* 2012). To determine if GluK1 receptors within the ACC are involved in itch-induced scratching, we implanted bi-lateral cannulae into the ACC of adult C57 mice (Wang *et al.* 2011) and microinjected UBP-302 (20 nM, 2 μM, 200 μM, or 3 mM) or vehicle (6% DMSO) 15 min prior to histamine application to the nape of the neck. As can be seen in Fig. 2a, cortical injections of a very low dose of UBP-302 (20 nM) yielded obvious decreases in scratching compared to vehicle. Increasing doses of UBP-302, to 2 μM and 200 μM resulted in more robust attenuation, decreasing scratching to similar levels observed in mice exposed to 3 mM cortical injections (vehicle: 128 ± 11 bouts, $n = 7$; UBP-302$_{20\ nM}$: 76 ± 9 bouts, $n = 6$; UBP-302$_{2\ \mu M}$ 63 ± 14 bouts, $n = 6$; UBP-302$_{200\ \mu M}$: 65 ± 5 bouts, $n = 6$; UBP-302$_{3\ mM}$: 62 ± 8 bouts $n = 7$). A one-way ANOVA revealed that all 4 doses of cortical UBP-302 injections significantly reduced scratching ($F_{6,36} = 6.6$, $p < 0.001$). In contrast, no differences in response latencies were observed in the hot plate test across all four doses of cortical UBP-302 injections, compared with cortical vehicle injections (Fig. 2b), indicating that acute nociceptive behavior is unaffected by pharmacological cortical GluK1 subunit antagonism, and that cortical injections of UBP-302 do not have a broad non-specific effect of suppressing behaviors related to sensory stimuli. We next sought to confirm that the effects of cortical UBP-302 were indeed indicative of cortical KA receptor modulation of scratching, and not because of a general effect of behavior suppression by changes in ACC excitability. To test this, we exposed C57 mice to cortical injections of AP-5, an NMDA receptor antagonist, and CNQX, an AMPA and KA receptor antagonist, and observed the effects on histamine-induced scratching. Remarkably, ACC injections of AP-5 had no effect on behavioral scratching, whereas CNQX injections yielded significant reductions in scratching (saline: 103 ± 12 bouts, $n = 7$; AP-5: 105 ± 12 bouts, $n = 6$; CNQX: 62 ± 9 bouts, $n = 6$; Fig. 2c). Accordingly, UBP-302 (3 mM) ACC microinjections also induced robust reductions in scratching in response to cowhage (vehicle: 113 ± 6 bouts, $n = 7$; UBP-302$_{3\ mM}$: 51 ± 10 bouts, $n = 7$; $t = 5.3$, $p < 0.001$; Fig. 2d). These results clearly demonstrate that

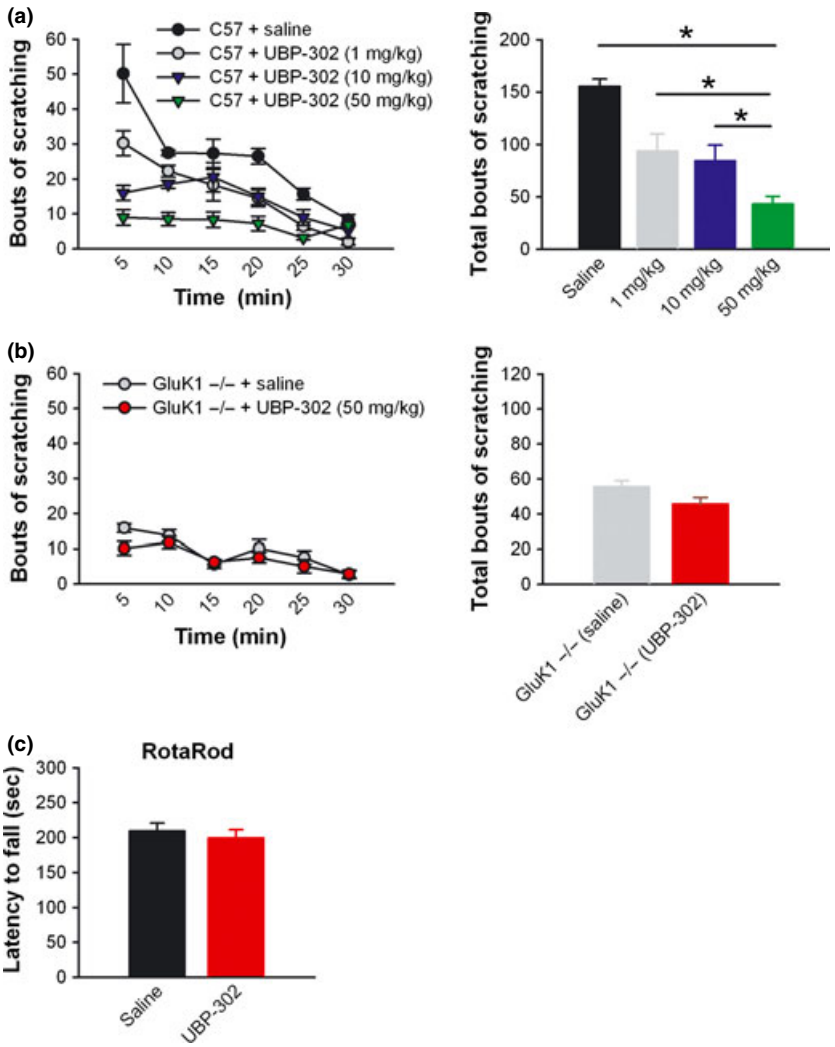


Fig. 1 Pharmacological inhibition of GluK1 reduces scratching. (a) Intraperitoneal (i.p.) injections of UBP-302 (1/10/or 50 mg/kg) or saline were administered to mice 30 min prior to histamine application. UBP-302 attenuated scratching behavior compared to saline in a dose dependent manner. C57 mice exposed to saline displayed significantly more scratching behavior compared to all three doses of UBP-302, and mice exposed to the larger dose of 50 mg/kg showed significantly less scratching than those exposed to lower doses of 1 and 10 mg/kg. No differences were detected in scratching levels between mice exposed to 1 or 10 mg/kg of UBP-302. (b) GluK1$^{-/-}$ mice exposed to UBP-302 (50 mg/kg) and GluK1$^{-/-}$ mice exposed to saline displayed similar scratching behavior in response to histamine. (c) C57 mice exposed to UBP-302 (50 mg/kg, i.p.) also showed normal motor function in the RotaRod test compared with C57 mice exposed to saline. Please note that C57 wild-type mice used in this Figure are inbred C57 mice while the GluK1$^{-/-}$ mutant mice were bred as a separate homozygous colony. (*$p < 0.001$).

inhibition of GluK1-containing KA receptors in the ACC can reduce scratching induced by distinct classes of itching stimuli.

Contribution of GluK1 to pruritogen-induced c-Fos expression in the ACC

Although recent human imaging studies have reported that itch corresponds with ACC activity, no animal study has investigated cortical activity in response to itch. Previous studies investigating spinal mechanisms of itch in mice have used the expression of the immediate early gene *c-fos* as a measure of recent neuronal activity (Akiyama *et al.* 2009). Similarly, studies investigating the role of the ACC in emotional behaviors have also interpreted c-Fos expression as indicative of recent neuronal involvement (Han *et al.* 2003; Frankland *et al.* 2004). As can be observed in Fig. 3b, histamine application yielded robust c-Fos immunopositive expression in the ACC of C57 mice, but only slight expression in the ACC of GluK1$^{-/-}$ mice. The expression patterns were significantly different in layers II/III (C57: 32 ± 2 cells, $n = 3$; GluK1$^{-/-}$: 6 ± 1 cells/1 × 10^5 μm^2, $n = 3$, $t = 11.6$, $p < 0.001$) and layers V/VI (C57: 30 ± 4 cells, $n = 3$; GluK1$^{-/-}$: 7 ± 1 cells/1 × 10^5 μm^2, $n = 3$, $t = 6.3$, $p = 0.003$; Fig. 3c–d). Saline application induced a small amount of c-Fos expression that was indistinguishable between groups (Fig. 3e–g). The c-Fos-positive neurons were mostly distributed in layers II/III and deeper layers V/VI in both sides of the ACC, although a few cells were also observed in layer I. These observations clearly show that ACC neurons are activated in correspondence with pruritogen-induced scratching, and suggest that GluK1 activity is involved in this activation; however, please note that ACC slices were collected from C57 wild-types and GluK1$^{-/-}$ mutant mice that were bred separately.

Pruritogen-induced scratching corresponds with alterations of excitatory transmission in ACC pyramidal neurons

Given the attenuating effects on scratching yielded through ACC microinjections of UBP-302, we next decided to employ *in vitro* whole cell patch clamp recordings to

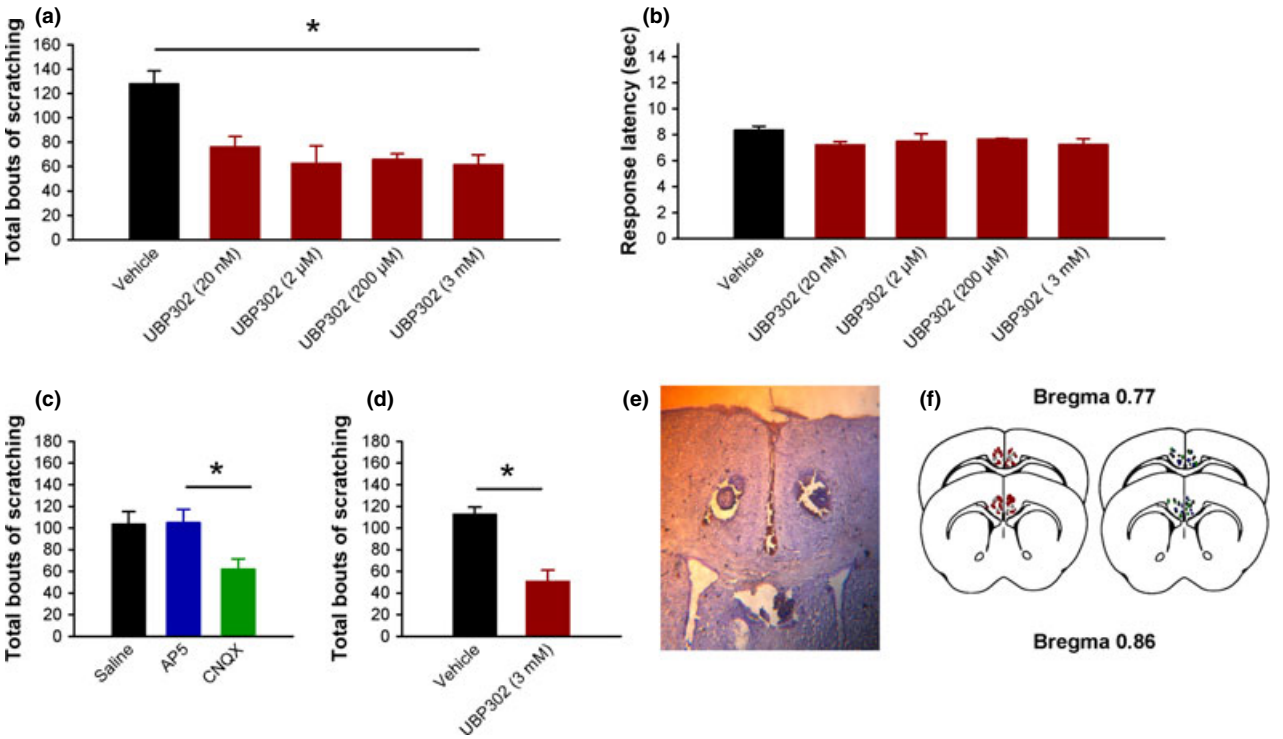


Fig. 2 Pruritogen-induced scratching involves GluK1- containing KA receptors in the anterior cingulate cortex (ACC). (a) ACC microinjections were performed 15 min prior to testing. Mice were randomly assigned to different doses of UBP-302 (20 nM, 2 μM, 200 μM, or 3 mM) or vehicle (6% DMSO) prior to intradermal histamine injections. ACC injections of UBP-302 (20 nM) yielded significant decreases in histamine-induced scratching compared to vehicle, which became more robust with increasing doses (2 μM, 200 μM, and 3 mM). All doses of UBP-302 resulted in statistically significant reductions of scratching. (b) Hot plate test results show that all four doses of intra-ACC UBP-302 injections corresponded to similar response latencies to vehicle injections. (c) Intra-ACC injections of AP-5, an NMDA receptor antagonist, had no effect on behavioral scratching, whereas CNQX, a KA and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptor antagonist significantly reduced scratching. (d) ACC injections of UBP-302 (3 mM) significantly decreases cowhage-induced scratching compared to vehicle injections. (e) Sample coronal slice with injection sites identified via post-mortem ink injections. (f) Representations of observed injection sites for UBP-302 and vehicle injections (left), and AP-5, CNQX, and saline injections (right). (*$p < 0.02$).

investigate ACC neuronal activity in response to itching stimuli. Recordings were performed on visually identified pyramidal cells in layers II/III of adult ACC slices extracted from mice 30 min after receiving either intradermal injections of histamine (250 μg/10 μL) or saline to the nape of the neck. We first focused on spontaneous excitatory post-synaptic currents (sEPSCs) and observed a marked increase in frequencies (Histamine: 11.2 ± 2.0, $n = 11$; Saline: 5.6 ± 2.2, $n = 11$, $t = 2.4$, $p < 0.05$; Fig. 4a–c) and amplitudes (Histamine: 9.1 ± 1.0 Hz, $n = 11$; Saline: 7.1 ± 0.3, $n = 11$, $t = 86$, $p < 0.01$; Fig. 4a–c) of sEPSCs. Remarkably, bath application of UBP-302 attenuated sEPSCs only from neurons obtained from mice exposed to histamine, but not in neurons of mice exposed to saline (Histamine: Pre-UBP-302: 95.1 ± 3.7% of baseline, $n = 7$; Post-UBP-302: 67.6 ± 5.3% of baseline, $n = 7$, $t = 8.7$, $p < 0.001$) (Saline: Pre-UBP-302: 97.9 ± 3.7% of baseline, $n = 6$; Post-UBP-302: 90.6 ± 2% of baseline, $n = 6$, $t = 8.7$, $p = 0.09$). Taken together, these results suggest that histamine-induced scratching corresponds with increases spontaneous excitatory transmissions in the ACC.

We recently showed that glutamate mediates excitatory transmission in itch-activated dorsal horn neurons in mice, whereby CNQX, a blocker of both AMPA and KA receptors blocked eEPSCs in cells expressing c-Fos (Koga *et al.* 2011). We thus next investigated if itch-activated ACC neurons received glutamatergic input. We used adult transgenic mice in which the expression of GFP is controlled by the promoter of the *c-fos* gene (Barth *et al.* 2004; Clem *et al.* 2008; Li *et al.* 2010), and performed whole cell patch clamp recordings in neurons recently activated by itch. Similar to our above immunostaining results, we observed many c-Fos immunopositive neurons in the ACC of transgenic mice after intradermal histamine injections, and recordings were performed on FosGFP-positive or negative pyramidal cells in

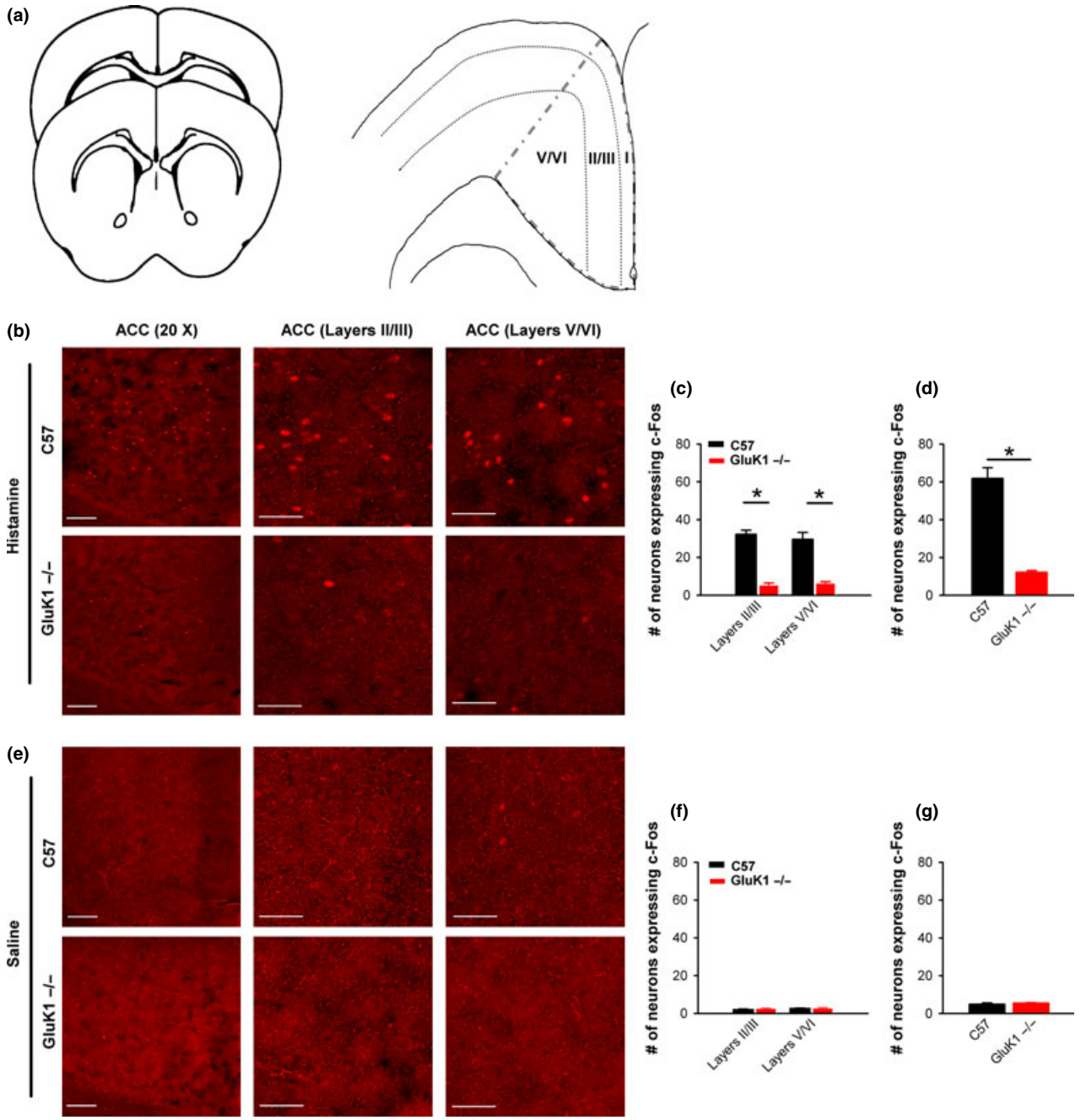


Fig. 3 Pruritogen-induced c-Fos expression in the anterior cingulate cortex (ACC) is reduced in GluK1$^{-/-}$ mice. (a) Non-serial coronal sections (4/mouse) containing the ACC were taken from mice 90 min post-application of histamine, or saline to the nape of the neck. (b) Slices were observed for c-Fos immunostaining in response to histamine. (c–d) c-Fos immunostaining was significantly reduced in all layers of GluK1$^{-/-}$ mice in response to histamine. (e) Slices were observed for c-Fos immunostaining in response to saline. (f–g) c-Fos immunostaining was similar in all layers of the ACC in C57 and GluK1$^{-/-}$ mice. Images of the ACC were taken at 20 X magnification (left column), and images of layers II/III (middle column) and layers V/VI (right column) were taken at 60 X magnification. Please note that C57 wild-type mice used in this Figure are inbred C57 mice while the GluK1$^{-/-}$ mutant mice were bred as a separate homozygous colony. Scale bars represent 100 μm for the left column and 50 μm for middle and right columns. (*$p < 0.02$).

layers II/III of the ACC from mice exposed to intradermal injections of histamine (Figure S3a). Histamine application yielded observable sEPSCs in FosGFP positive cells which were completely blocked by CNQX (25 μM) (Figure S3b) (Frequency: control: 5.8 ± 1.9 Hz, $n = 3$; CNQX: 0.2 ± 0.2 Hz, $n = 4$, $t = 3.4$, $p = 0.02$) (Amplitude: con-

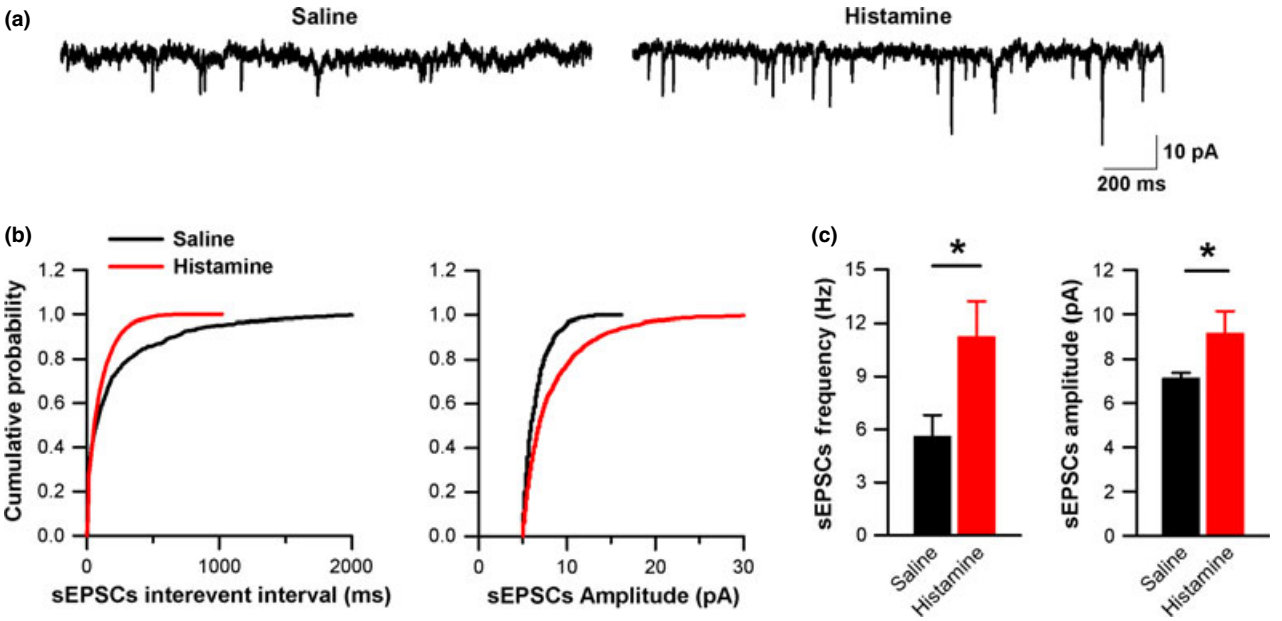


Fig. 4 Itching stimuli corresponds with alterations of spontaneous excitatory post-synaptic currents (sEPSCs) in anterior cingulate cortex (ACC) pyramidal neurons. (a) Representative sample traces of sEPSCs from ACC pyramidal neurons of mice exposed to intradermal injections of saline (left) or histamine (right). (b–c) Histamine injections induced a significant increase in frequencies and amplitudes of eEPSCs of layer II/III ACC pyramidal neurons. (*$p < 0.05$).

trol: 14.7 ± 1.6 pA, $n = 3$; CNQX: 2.8 ± 2.8 pA, $n = 4$, $t = 3.3$, $p = 0.02$). Histamine application also yielded observable sEPSCs in FosGFP-negative cells, which were also completely blocked by CNQX (Frequency: control: 7.0 ± 1.5 Hz, $n = 5$; CNQX: 0.2 ± 0.1 Hz, $n = 5$, $t = 40$, $p < 0.01$) (Amplitude: control: 9.9 ± 1.1 pA, $n = 5$; CNQX: 1.1 ± 0.4 pA, $n = 5$, $t = 7.9$, $p < 0.01$; Figure S3d). These data indicate that pruritogen-activated pyramidal neurons in the ACC receive glutamatergic input; however, we cannot rule out the possibility that non-c-Fos expressing cells may also be involved in itch, or that repetitive scratching also activates c-Fos within the ACC. Moreover, although previous reports have shown that some ACC neurons respond to noxious stimuli on any part of the body surface (Vogt 2005), it is possible that FosGFP-negative cells may respond to histamine but have receptive fields beyond the histamine injection spot.

Itching stimulation does not recruit post-synaptic KA receptor activity in ACC pyramidal neurons

Next, we wanted to determine whether post-synaptic KA receptor function is altered in mice exposed to histamine or saline, and thus recorded post-synaptic KA mediated currents in the ACC. In the presence of picrotoxin (100 μM) and the selective NMDA receptor antagonist AP-5 (50 μM), AMPA/KA mediated eEPSCs were recorded from layer II/III neurons during stimulation by a bipolar tungsten electrode placed in layer V/VI of the ACC (Wu *et al.* 2005). The single-pulse stimulation yielded AMPA/KA mediated EPSCs in ACC neurons from mice exposed to saline or histamine (Fig. 5a). To isolate KA currents, we added the selective and potent AMPA receptor antagonist, GYKI 53655 (100 μM) in the bath solution for 10 min, which rapidly reduced the amplitude of the observed EPSCs. The small, residual EPSCs that persisted in the presence of GYKI 53655 represented isolated KA currents, and did not differ between mice exposed to saline or histamine (Histamine: 7.8 ± 1.3%, $n = 6$; Saline: 6.9 ± 2.0%, $n = 6$, $t = 32.0$, $p = 0.31$; Fig. 5b). This suggests that histamine does not alter post-synaptic KA receptor function in the ACC. We further studied repetitive stimulation induced KA functions, as brief repetitive impulse trains robustly facilitate KA receptor-mediated EPSCs in most synapses (Castillo *et al.* 1997; Wu *et al.* 2005). Using repetitive stimulation paradigms, we determined the summation properties of KA receptor-mediated synaptic responses in the ACC of mice exposed to itch in response. We found that increases in number of shocks presented (single, 5, 10, and 20 shocks at 200 Hz) yielded obvious increases of KA receptor-mediated EPSC amplitudes in saline and histamine groups (Fig. 5c), with similar resulting amplitudes between both groups (Fig. 5d). Similarly, analysis of the input–output relationships of KA receptor-mediated EPSCs, measuring the EPSCs amplitude (output) as a function of the afferent stimulus intensity (input), showed almost identical slopes. Together, these data suggest that exposure to itching stimuli does not significantly

Fig. 5 Itching stimuli does not enhance post-synaptic KA receptor activity in anterior cingulate cortex (ACC) pyramidal neurons. (a) In the presence of picrotoxin (100 μM) and the selective NMDA receptor antagonist AP-5 (50 μM), single pulse stimulation yielded 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA)/KA mediated excitatory post-synaptic currents (EPSCs) (top) and application of GYKI 53655 (100 μM) for 10 min in the bath solution reduced the amplitude of the observed EPSCs (bottom). (b) The residual EPSCs represented isolated KA currents, and did not differ between mice exposed to saline or histamine. (c) Representative sample traces of currents induced by repetitive shocks (5, 10, and 20) at 200 Hz. (d) Increases of KA receptor-mediated EPSC amplitudes were similar in neurons from mice exposed to saline or histamine. (e) Representative sample traces of currents induced by increasing stimulus intensities. (f) Input–output relationships of KA receptor-mediated EPSCs were also similar.

change post-synaptic KA mediated synaptic efficacy in ACC pyramidal neurons.

Pruritogen-induced ACC GluK1 activity modulates GABAergic transmission

What could be the possible mechanism driving the effect of GluK1 on pruritogen-induced scratching? One possibility is that itching-stimuli-induced ACC GluK1 receptor activity may modulate GABAergic transmission, as we have previously observed that in the ACC, activation of KA receptor GluK1 subunits can mediate GABAergic transmission (Wu *et al.* 2007). Using whole cell patch clamp, we recorded eEPSPs from pyramidal ACC neurons of mice exposed to histamine or saline 30 min prior, and analyzed amplitudes before and after bath applications of UBP-302 (10 μM) for 15 min. Without blocking $GABA_A$ receptors by picrotoxin (100 μM), the amplitude of eEPSPs in ACC slices from mice that had been exposed to histamine were significantly attenuated by bath application of UBP-302 (pre-UBP-302: 100 ± 1.2% of baseline; post-UBP-302: 81.6 ± 1.2% of baseline, $n = 8$, $t = 17.3$, $p < 0.001$; Fig. 6a and e) . In contrast, in recordings from mice exposed to saline, amplitudes of eEPSPs were not diminished, and were even slightly elevated, by bath application of UBP-302 (pre-UBP-302 100 ± 1.2% of baseline; post-UBP-302: 107.1 ± 1.3% of baseline, $n = 8$; Fig. 6a and e). Furthermore, decay time of the normalized EPSPs from histamine exposed mice was significantly shortened by UBP-302, but not in saline exposed mice (pre-UBP-302 39.4 ± 4.7 ms; post-UBP-302: 25.5 ± 5.2 ms, $n = 8$, $p < 0.05$; Fig. 6b). Remarkably, the EPSP attenuating effect of UBP-302 on neurons from mice exposed to histamine was completely blocked if picrotoxin (100 μM), a potent $GABA_A$ receptor antagonist, was included in the bath solution (pre-UBP-302: 100 ± 1.2% of baseline; post-UBP-302: 97.2 ± 2.2% of baseline, $n = 6$, $t = 1.3$, $p = 0.198$; Fig. 6c and e). These observations indicate that pruritogen-induced KA receptor GluK1 subunit activity modulates GABAergic transmission.

ACC GluK1 receptors modulate behavioral responses to acute inflammatory pain

Itch and pain sensation both induce robust behavioral responses, and interactions between itch and pain have been well documented (Ikoma *et al.* 2006; Patel and Dong 2010). Recent evidence shows that some thalamic projecting neurons may be activated by both pain and itching stimuli (Davidson *et al.* 2012). The ACC receives robust projections from the thalamus and neuroimaging studies have shown that it is activated by allergens and noxious stimuli (Davidson and Giesler 2010). It is therefore possible that ACC neurons activated by itching stimuli may also be activated by noxious stimuli, and as we have shown that GluK1 subunits within the ACC modulate pruritogen-induced scratching behavior, it is possible that they also modulate nociceptive behavior. To test this possibility, we first assessed behavioral response latencies to acute physiological pain in inbred C57 wild-type mice and $GluK1^{-/-}$ mutant mice that were bred as a separate homozygous colony. Behavioral response latencies were similar between groups in response to the hot plate (C57: 5.8 ± 0.29 s, $n = 6$; $GluK1^{-/-}$: 5.1 ± 0.24 s, $n = 5$, $t = 1.8$, $p = 0.11$; Figure S4a) and tail-flick tests (C57: 6.2 ± 0.3 s, $n = 6$; $GluK1^{-/-}$: 5.9 ± 0.6 s, $n = 5$, $t = 0.3$, $p = 0.74$; Figure S4b), indicating that KA receptor GluK1 subunits do not mediate acute physiological pain. In contrast, we have previously shown that $GluK1^{-/-}$ mice display deficits in formalin (5%) induced nociceptive behavior (Ko *et al.* 2005), and that formalin (5%) injection is accompanied by robust c-Fos expression in ACC neurons (Wei *et al.* 2001). We thus compared nociceptive responses to hindpaw injections of formalin (5%) in three groups of mice: adult $GluK1^{-/-}$ mice, adult C57 mice exposed to bilateral ACC microinjections of UBP-302 (3 mM), and adult naïve C57 mice. In accordance to previous reports, $GluK1^{-/-}$ mice exhibited reduced nociceptive behavior compared to C57 mice (Figure S4c). Remarkably, this reduction was replicated in mice exposed to ACC microinjections of UBP-302. Interestingly, separately bred $GluK1^{-/-}$ mice and C57 mice with ACC injections of UBP-302 displayed similar nociceptive behavior as C57 mice in the first 10 min (Phase 1) (C57: 59.4 ± 11.9 s, $n = 6$; $GluK1^{-/-}$ 58.9 ± 9.6 s, $n = 6$, UBP-302: 61.7 ± 7.3 s, $n = 6$; $F_{(2,\ 15)} = 0.02$, $p = 0.98$; Figure S4d). During phase two (10–60 min), however, $GluK1^{-/-}$ mice and C57 mice with ACC injections of UBP-302, showed a robust reduction in nociceptive behavior compared to naïve C57 mice, and this attenuation was comparable between $GluK1^{-/-}$ and UBP-302 treated C57 mice (C57: 422 ± 66.2 s, $n = 6$; $GluK1^{-/-}$ 180.5 ± 12.9 s, $n = 6$, UBP-302: 189.1 ± 21.5 s, $n = 6$; $F_{(2,\ 15)} = 11.2$,

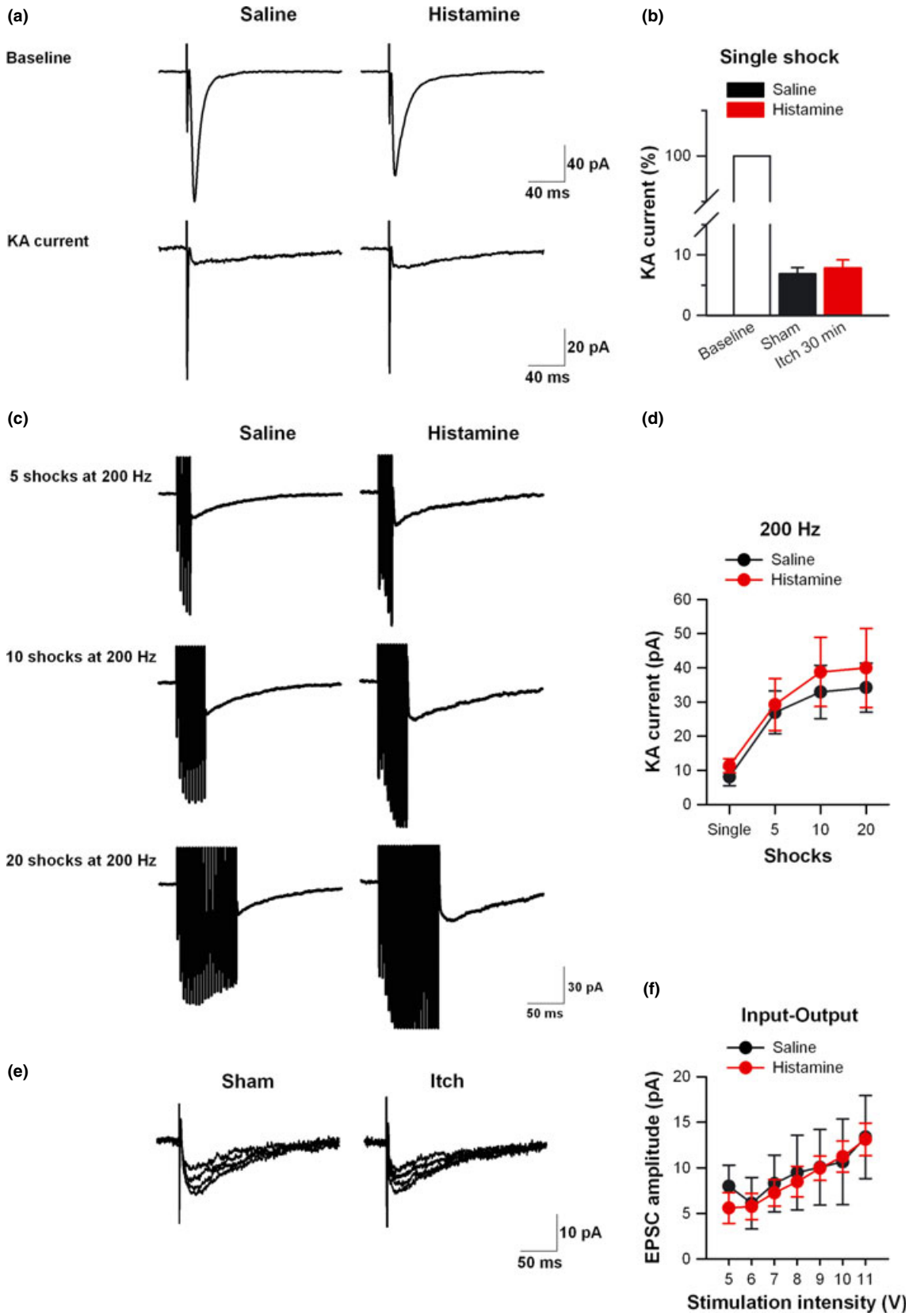

(a)
Saline
Histamine
Baseline
40 pA
40 ms
KA current
20 pA
40 ms
(b)
Single shock
Saline
Histamine
KA current (%)
100
10
0
Baseline
Sham
Itch 30 min
(c)
Saline
Histamine
5 shocks at 200 Hz
10 shocks at 200 Hz
20 shocks at 200 Hz
30 pA
50 ms
(d)
200 Hz
Saline
Histamine
KA current (pA)
60
50
40
30
20
10
0
Single
5
10
20
Shocks
(e)
Sham
Itch
10 pA
50 ms
(f)
Input-Output
Saline
Histamine
EPSC amplitude (pA)
20
15
10
5
0
5 6 7 8 9 10 11
Stimulation intensity (V)

Fig. 6 Pruritogen-induced anterior cingulate cortex (ACC) GluK1 activity modulates GABAergic transmission. We recorded the evoked potentials from layers II/III in response to stimulations of layer V/VI, and recorded evoked excitatory post-synaptic potentials (eEPSPs) amplitudes before and after bath applications of UBP-302 (10 μM). (a) Representative sample traces of eEPSPs recorded from layer II/III ACC pyramidal neurons extracted from mice 30 min after exposure to saline (top) or histamine (bottom). In the absence of picrotoxin (100 μM), a potent $GABA_A$ receptor antagonist, the amplitude of eEPSPs were significantly reduced in recordings from ACCs of mice that had been exposed to histamine, but not in recordings from the ACCs of mice exposed to saline. (b) Open circles indicate EPSP decay time in the absence of UBP-302 for histamine (open red) and saline (open black) exposed mice. The decay time of the normalized EPSPs from histamine exposed mice was significantly shortened by UBP-302 (solid red circles), but not in saline exposed mice (solid black circles). (c) Remarkably, in the presence of picrotoxin (100 μM), the attenuating effect of UBP-302 was blocked. (d) The effect of UBP-302 on the decay time of KA receptors from histamine exposed mice was also blocked by pricrotoxin. (e) Summarized data of the amplitudes of eEPSPs in saline and histamine group with or without picrotoxin. (*$p < 0.05$).

$p = 0.001$; Figure S4d). These results indicate that KA activity within the ACC modulates licking responses induced by peripheral inflammatory stimuli, and suggest that overlapping cortical mechanisms can modulate behavioral responses in response to itch and inflammatory pain; although it is important to note that intradermal formalin injections have been shown to induce scratching (Ross *et al.* 2010; Patel *et al.* 2011), and thus the observed licking behavior may represent formalin induced itch.

Discussion

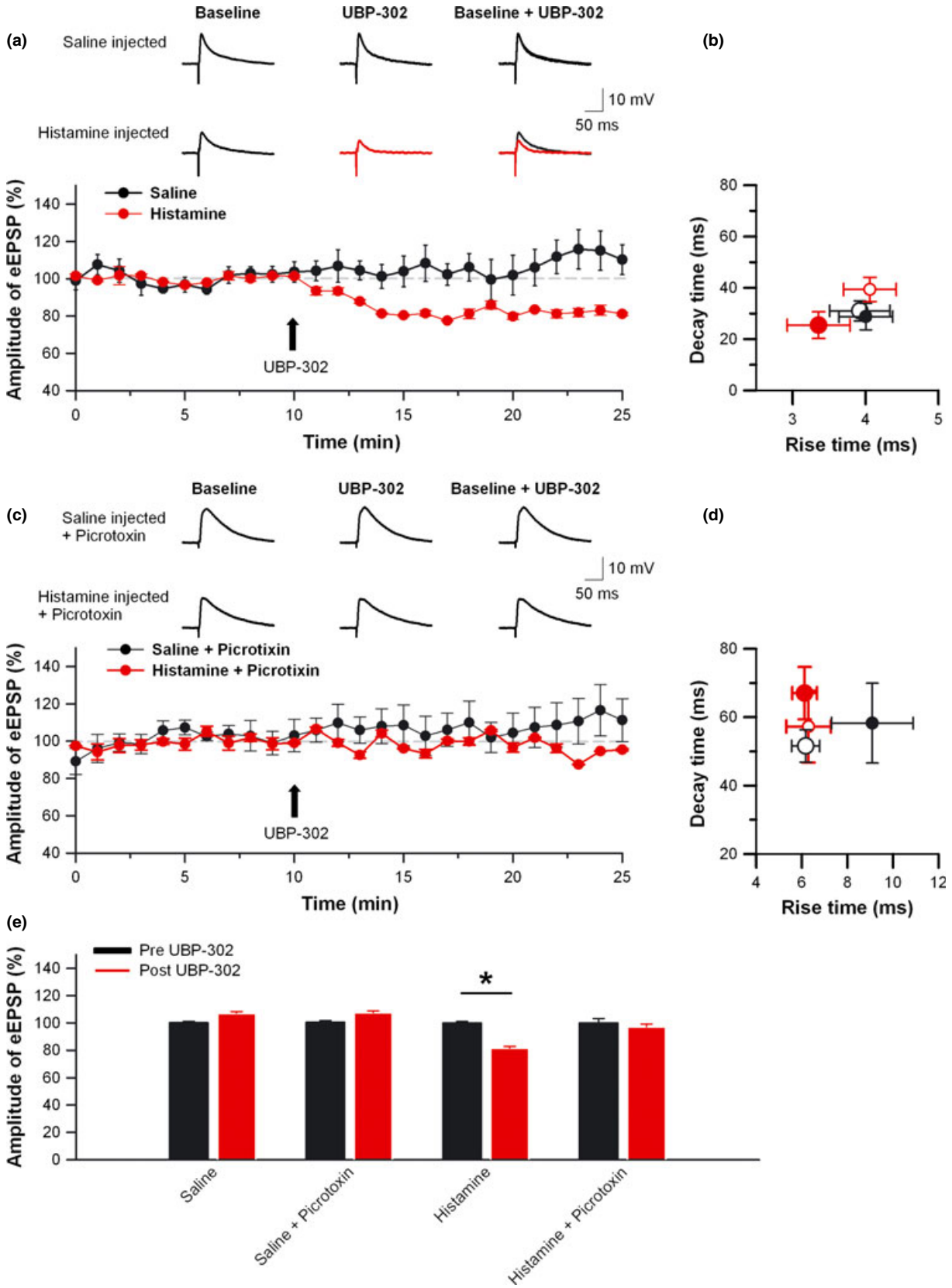
This study shows that cortical GluK1-containing KA receptors modulate behavioral responses to itch and inflammatory pain. Our results demonstrate that genetic deletion (preliminary data) or pharmacological inhibition of GluK1 subunits attenuates scratching induced by histamine and non-histamine-dependent pruritogens. Furthermore, we show that inhibition of GluK1 specifically within the ACC also reduces scratching, and that pruritogen-induced neuronal activity in the ACC is reduced in $GluK1^{-/-}$ mice. Accordingly, through whole cell patch clamp recordings, we show that pruritogen-induced scratching corresponds with enhanced glutamatergic excitatory transmission in layers II/III in the ACC, and engages GluK1 mediated inhibitory circuitry. Lastly, we show that pharmacological inhibition of GluK1 subunit activity within the ACC also reduces behavioral responses to inflammatory pain.

Cortical contribution to itching-induced scratching

Previous human imaging studies have shown ACC activity in correspondence with itch (Ikoma *et al.* 2006); however, no animal study had determined whether cortical activity corresponds with modulation of pruritogen-induced scratching. In this study, we present evidence of cortical contribution to scratching in mice by showing that intra-ACC injections of UBP-302 robustly attenuate itching-stimuli-induced scratching. The connections that are formed by the ACC place it in a great position to affect behavioral responses induced by peripheral stimuli. For example, *in vivo* studies show that some ACC neurons respond to noxious stimulation on any part of the body surface (Vogt 2005). Importantly, the ACC receives robust projections from the thalamus (Lee *et al.* 2007), forms multiple reciprocal connections with other cortical areas (Vogt 2005), including the motor cortex, (Vogt *et al.* 2005; Leknes *et al.* 2007), and contributes to descending facilitation of spinal cord transmission (Calejesan *et al.* 2000). Accordingly, we observed that scratching behavior corresponded to ACC activity, and scratching has been shown to correspond with motor cortex activity (Sirota *et al.* 2006). Similarly, neuroimaging studies have observed co-activation of the ACC, supplementary, and pre-motor areas in correspondence with the urge to scratch in humans (Hsieh *et al.* 1994; Leknes *et al.* 2007). Peripheral pruritogen stimulation may thus engage KA receptor modulated ACC projections to the motor cortex (Vogt *et al.* 2005; Leknes *et al.* 2007), or KA receptor modulated descending facilitation of spinal cord transmission (Calejesan *et al.* 2000). Pure involvement of the ACC in motor responses and not sensation of itch however seem unlikely, as electrical stimulation (Tang *et al.* 2005) or chemical activation (Johansen and Fields 2004) of the ACC do not induce any scratching–like motor responses. Unfortunately, it is impossible to separate the sensation of itch from the behavioral response in a mouse model. For instance, although Elizabethean collars could be used to prevent mice from successfully scratching, in our experience mice still attempt to scratch and thus still perform the same hind paw behavioral movements.

Kainate GluK1 receptors in itch and pain

Scratching can be classified as a nocifensive behavior, and whether itch and pain share common mechanisms is a hotly debated topic (Papoiu *et al.* 2012). Whereas some studies have identified itch-specific spinal mechanisms (Schmelz *et al.* 1997; Sun and Chen 2007), others have highlighted robust interactions between both experiences (Ikoma *et al.* 2006; Yosipovitch *et al.* 2008; Davidson and Giesler 2010), suggesting they share similar mechanisms. Our data indicate that cortical KA receptors are involved in itch- and inflammatory pain-induced nocifensive behaviors. Specifically, we observed that in addition to attenuating scratching, pharmacological inhibition of GluK1 within the ACC also reduced formalin-induced behavioral responses. Thus, although pain


(a)
Baseline
UBP-302
Baseline + UBP-302
Saline injected
Histamine injected
10 mV
50 ms
Saline
Histamine
Amplitude of eEPSP (%)
UBP-302
Time (min)
(b)
Decay time (ms)
Rise time (ms)
(c)
Saline injected
+ Picrotoxin
Histamine injected
+ Picrotoxin
Saline + Picrotixin
Histamine + Picrotixin
(d)
(e)
Pre UBP-302
Post UBP-302
*
Saline
Saline + Picrotoxin
Histamine
Histamine + Picrotoxin

and itch may have distinct spinal mechanisms (Patel and Dong 2010), our data show that cortical KA can modulate itching and inflammatory stimuli induced nociceptive behaviors. Accordingly, various neuroimaging and anatomical studies have shown evidence that several nuclei likely receive projections from both painful and itching stimuli (Jinks and Carstens 2000; Leknes *et al.* 2007; Yosipovitch *et al.* 2008; Davidson and Giesler 2010), and that some thalamic projecting neurons may be activated by both pain and itch (Davidson *et al.* 2012). As itch and pain are subjectively different experiences and elicit distinct responses (Shimada and LaMotte 2008), it is possible that these differences may be coded at the neuronal population level – that is some neurons are recruited by painful stimuli while others are by itching stimuli. Importantly, we observed that behavioral responses to acute physiological pain were unaltered by cortical GluK1 antagonism, demonstrating that cortical injections of UBP-302 do not have a general effect of suppressing nociceptive behavior. It is important to note, however, that although our pharmacological ACC microinjection experiments provide direct evidence for the requirement of cortical GluK1 in pruritogen-induced scratching and pain-induced licking, we cannot rule out possible spinal and peripheral contributions of KA receptors in these behaviors (Kerchner *et al.* 2001).

Cortical kainate GluK1 receptor mechanisms

We have shown that itching stimuli enhances glutamatergic transmission in the ACC, and cortical inhibition of GluK1-containing KA receptors robustly reduces pruritogen-induced scratching and formalin induced licking, but does not affect acute nociceptive behavior. Importantly, cortical injections of AP-5, an NMDA receptor antagonist had no effect on pruritogen-induced scratching; highlighting that attenuation of local excitability in the ACC is insufficient to decrease itching-induced behaviors. We have previously shown that pyramidal neurons of layers II/III in the ACC express post-synaptic KA receptors containing GluK1 subunits (Wu *et al.* 2005), and that these receptors mediate a small component of the post-synaptic excitatory current. In this study, however, we show that itching stimuli do not enhance post-synaptic KA receptor activity. We therefore propose a possible cortical mechanism whereby itching stimuli induces glutamatergic release at ACC synapses, activating post-synaptic AMPA receptors and pre-synaptic GluK1-containing KA receptors on inhibitory neurons, which in turn modulate GABAergic release (Fig. 7). We reach this conclusion from our evidence that pruritogen application enhances depression of post-synaptic excitatory potentials by UBP-302, indicating an increase in GluK1-containing KA receptor activity. We further found that this enhanced susceptibility is completely blocked by $GABA_A$ antagonism, indicating that GluK1 induced modulation by acting through GABAergic transmission. We have previously demonstrated that KA receptors-containing GluK1 subunits can modulate evoked GABAergic transmission within the ACC (Wu *et al.* 2007). This is consistent with several lines of evidence that suggest pre-synaptic KA receptors can modulate GABAergic inhibition in the mammalian brain (Lerma 2003). Our data

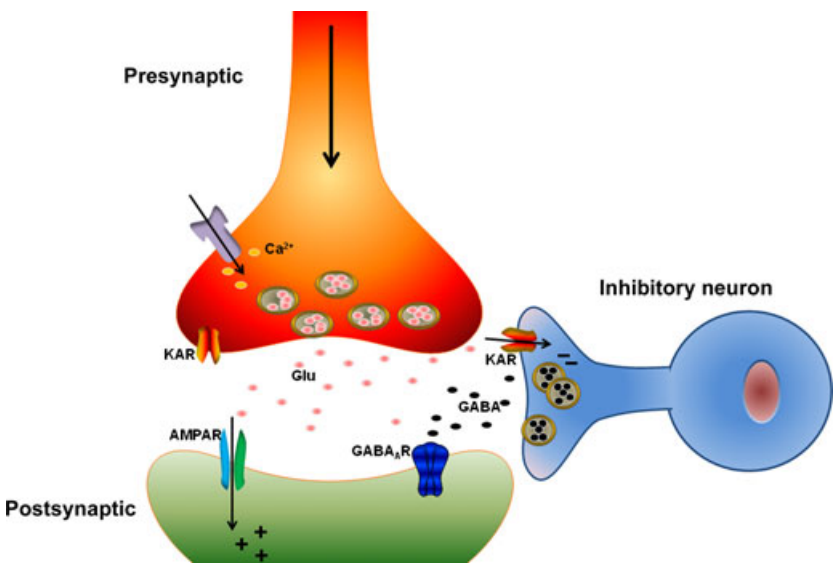


Fig. 7 Pruritogen dependent KA receptor modulation of evoked GABAergic transmission in the anterior cingulate cortex (ACC). A model of ACC synaptic modulation during pruritogen-induced scratching. Peripheral application of itching stimuli activates excitatory afferents projecting to ACC layer II/III pyramidal neurons. Activity-dependent pre-synaptic glutamate (Glu) release activates post-synaptic 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptors located on pyramidal neurons thereby enhancing the post-synaptic response. Simultaneously, pre-synaptic Glu can activate pre-synaptic kainate receptors (KAR) located on inhibitory neurons, thereby modulating GABA release and affecting the attenuation of the post-synaptic response.

show that cortical GluK1 activity is involved in pruritogen-induced scratching, and suggest that GluK1 modulation of evoked GABergic transmission is likely involved in the experience of itch. Further studies focusing on inhibitory neurons are needed to determine how KA receptors modulate GABAergic transmission during itch.

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We thank Dr. Feng Wei for helpful suggestions and Heather Kaang for help with behavior work. KK is supported through a post-doctoral fellowship from the Fragile-X research foundation of Canada; GD is supported by the E. Fidlar Ontario Graduate Scholarship. The authors declare no conflict of interest.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Preliminary evidence – contribution of KA receptor GluK1 subunits in pruritogen-induced scratching behavior.

Figure S2. GluK1$^{-/-}$ mice show reduced scratching compared with C57 and GluK1 WT littermates.

Figure S3. Recordings of sEPSCs from itching-stimuli activated ACC pyramidal neurons.

Figure S4. ACC GluK1 receptors modulate acute inflammatory pain.

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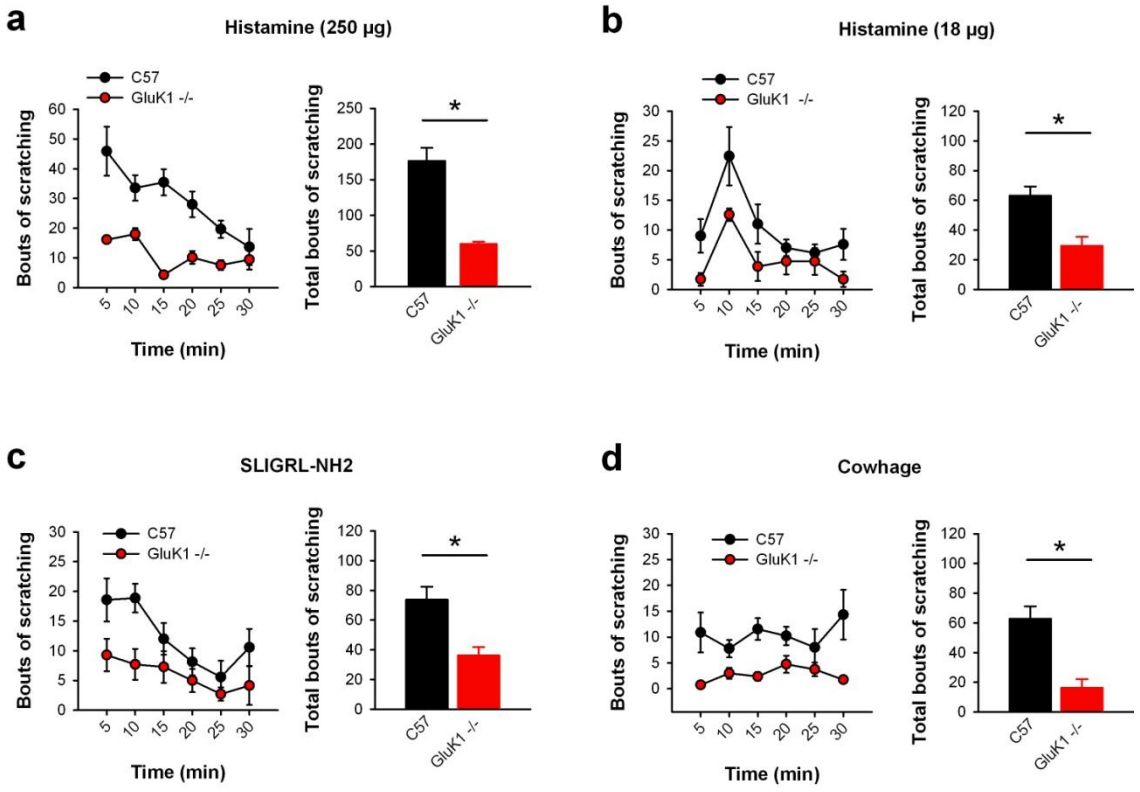
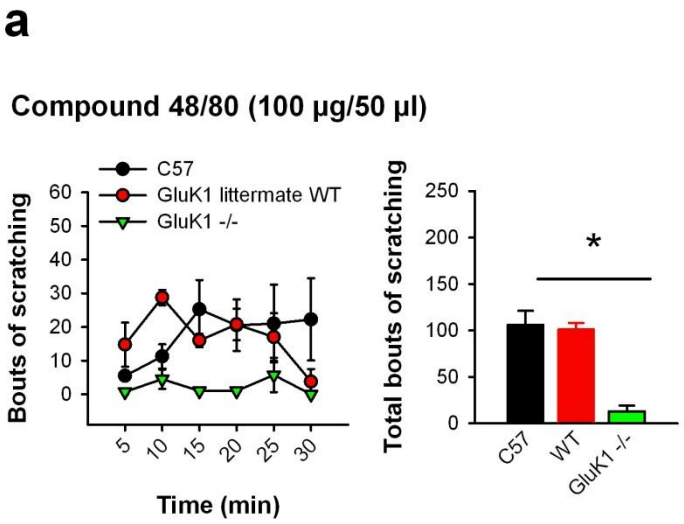
Supplementary Fig 1



Supplementary Figure 1: Preliminary evidence -- contribution of KA receptor GluK1 subunits in pruritogen-induced scratching behavior

Scratching behavior was observed in adult male mice for 30 min in response to itch-inducing stimuli. a) Intradermal application of histamine (250 µg/ 10 µl) into the nape of the neck induced robust scratching in C57 mice, but yielded significantly less scratching GluK1 $^{-/-}$ mice. Differences in scratching between the two groups were evident within 5 min post histamine application, but diminished throughout the test as C57 mice displayed time dependent reductions in scratching. b) Lower concentrations of histamine (18 µg /10 µl) yielded a similar phenotype. c) GluK1 $^{-/-}$ mice also showed significantly less scratching to PAR-2 stimulation by SLIGRL-NH_2 (25 µg / 10 µl) or d) application of cowhage spicules onto exposed skin on the neck. Cowhage induced scratching maintained similar levels throughout the 30 min observation period. Please note that C57 wild types used in this Figure are inbred C57 mice while the GluK1 $^{-/-}$ mutant mice were bred as a separate homozygous colony. (* $P < 0.02$).

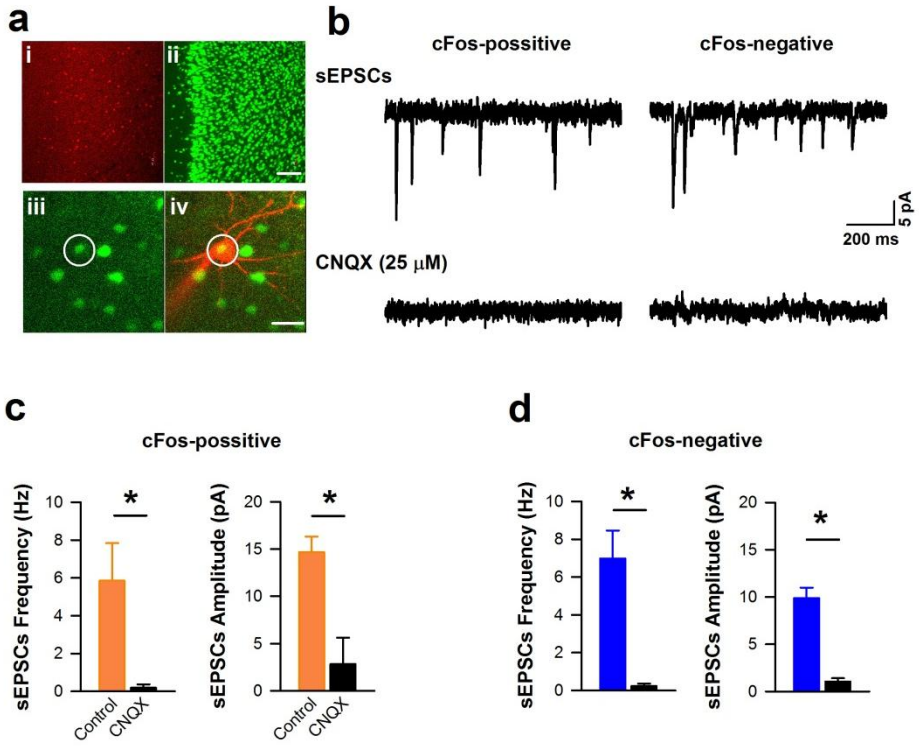
Supplementary Fig 2



Supplementary Figure 2: GluK1 -/- mice show reduced scratching compared with C57 and GluK1 WT littermates

a) GluK1 $^{-/-}$ mice displayed reduced scratching behavior after exposure to compound 48/80, whereas separately bred C57 and GluK1 WT littermates showed similar levels of scratching. (*$P < 0.001$)

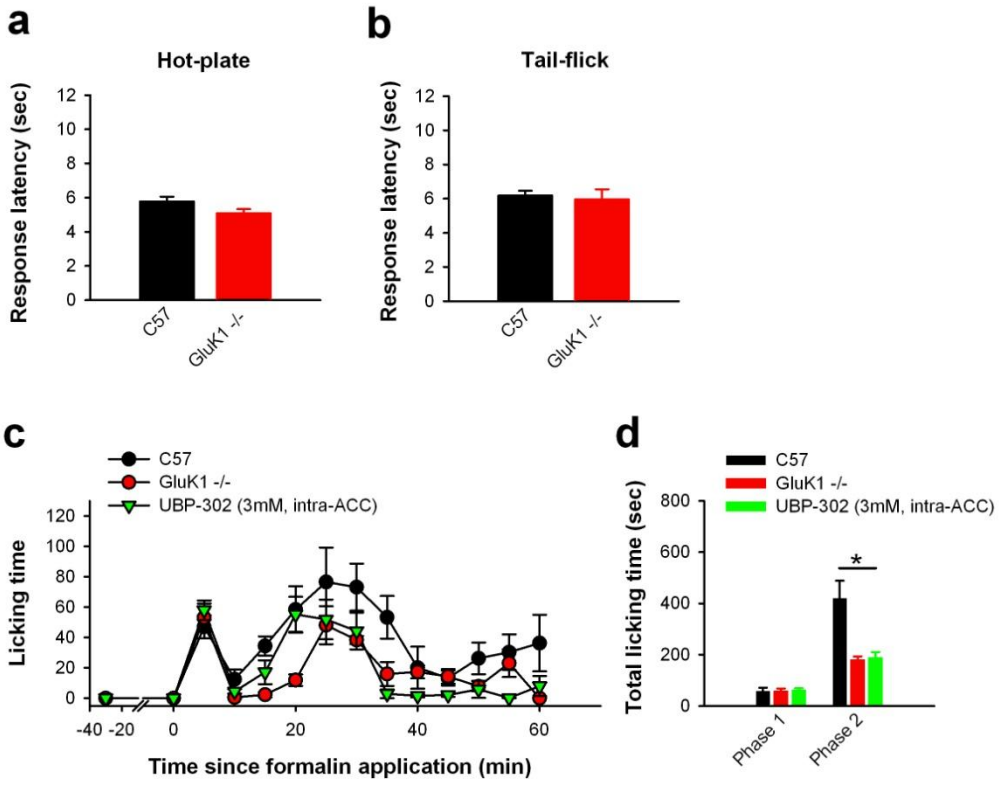
Supplementary Fig 3



Supplementary Figure 3: Recordings of sEPSCs from itching-stimuli activated ACC pyramidal neurons

a) (i) c-Fos expression and (ii) NeuN immunostainning of ACC neurons. (iii) FosGFP-expressing neurons in the ACC were recorded and labeled by Alexa fluor 594; (iv) yellow color indicates the overlap of GFP and Alexa fluor 594. Scale bar represents 20 μm. b) Sample traces of sEPSCs (top) recorded from FosGFP-possitive (left) and negative (right) pyramidal neurons of the ACC, which were blocked by bath application of CNQX (25 μM) (bottom). c) CNQX completely blocked sEPSCs of cFos-possitive and d) -negative neurons. (*$P < 0.05$).

Supplementary Fig 4



Supplementary Figure 4: ACC GluK1 receptors modulate acute inflammatory pain
a) GluK1 $^{-/-}$ mice displayed similar response latencies to C57 mice in the hot plate and b) tail flick assays. c) We exposed mice to left hindpaw injections of formalin (5%) and observed licking of the left hindpaw before and for 1 hr post application. d) During phase one (0 – 10 min), C57, GluK1 $^{-/-}$, and mice with ACC injections of UBP-302 (3mM) displayed similar nociceptive behavior. During phase two (10 – 60 min), GluK1 $^{-/-}$ mice and mice with ACC injections of UBP-302 (3mM) showed a robust reduction in nociceptive behavior compared to C57 mice. Licking behavior was comparable between GluK1 $^{-/-}$ and UBP-302 treated mice, and showed no significant difference throughout Phase 2. Please note that C57 wild types used in this Figure are inbred C57 mice while the GluK1 $^{-/-}$ mutant mice were bred as a separate homozygous colony. (*$P < 0.001$)