ROLE OF THE ANTERIOR CINGULATE CORTEX IN FEAR LEARNING AND SENSATION RELATED BEHAVIORS

by

Giannina Descalzi

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Giannina Descalzi

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Department of Physiology

University of Toronto

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Abstract

Neural activity within the brain underlies complex behavior that allows us to interact with our environment. The anterior cingulate cortex (ACC) is believed to mediate appropriate behavioral responses by integrating emotional and cognitive information about external stimuli. If this understanding is correct, then neural activity within the ACC must therefore correlate with behavioral output in response to external experience. The aim of this thesis is to bridge mechanisms identified in vitro with behaviors observed in vivo to determine the neural substrates of ACC mediated behavior. This thesis focuses on glutamatergic receptors that have been established as mediators of
excitatory transmission in the ACC. Through a combination of behavioral, pharmacological, biochemical, and electrophysiological methods, this thesis examined how behaviors observed in mouse models of fear learning, chronic pain, and itch correspond with in vitro observations of ACC neuronal activity. Three sets of experiments are presented. The first set investigated cortical LTP-like mechanisms, and assessed whether they could mediate fear learning. These sets of experiments provide in vivo evidence that trace fear learning requires rapid, NMDA receptor dependent, cortical AMPA receptor insertion. The second set of experiments investigated the contribution of forebrain CREB-mediated transcription in behavioral manifestations of chronic pain. These experiments show that forebrain overexpression of CREB is sufficient to enhance mechanical allodynia in animal models of chronic inflammatory or neuropathic pain. Lastly, the final set of experiments show that pruritogen-induced scratching corresponds with enhanced excitatory transmission in the ACC through KA receptor modulation of inhibitory circuitry. Through investigations of multiple behaviors linked to ACC activity, this thesis presents evidence that manifestations of behavior can be observed at the molecular level, and indicates that molecular mechanisms involved in ACC synaptic activity are a good target for translational research into pathological conditions that are related to abnormal ACC activity.
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A brief note on collaborations

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List of peer reviewed publications achieved throughout PhD

** indicates publication used in this thesis


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List of abbreviations

ACC Anterior cingulate cortex
AMPA α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate
CaMKIV Calcium/calmodulin-dependent protein kinase IV
CNQX 6-cyano-7-nitroquinoxaline-2, 3-dione disodium salt
CREB Cre-response element binding protein
CS Conditioned stimulus
DH Dorsal horn
DRG dorsal root ganglion
EPSC excitatory postsynaptic current
EPSP excitatory postsynaptic potential
GABA γ-Aminobutyric acid
IPSC inhibitory postsynaptic current
KA kainate
LTP Long-term potentiation
LTD Long-term depression
NMDA N-methyl-D-aspartate
GluN2A NMDA receptor subunit 2A
NR2B NMDA receptor subunit 2B
US Unconditioned stimulus
CHAPTER 1: INTRODUCTION

ANATOMICAL CONNECTIONS AND SYNAPTIC TRANSMISSION IN THE ANTERIOR CINGULATE CORTEX
CHAPTER 1: INTRODUCTION

ANATOMICAL CONNECTIONS AND SYNAPTIC TRANSMISSION IN THE ANTERIOR CINGULATE CORTEX

1.1 Overview

This chapter reviews literature characterizing the anterior cingulate cortex (ACC). It provides an overview of the anatomical connections with other parts of the central nervous system (CNS), and summarizes molecular mechanisms mediating synaptic transmission within the ACC. This section highlights critical cellular and molecular findings which will provide the basis for the three sets of experiments presented in this thesis. A key concept is that cellular activity within the brain underlies complex behavior and mental states. Although various interesting discussions highlight the mind-brain gap, it is understood that the complexities of human experience, both cognitively and behaviorally, are driven by equally complex brain activity. As will be apparent throughout the literature and data presented in this thesis, much evidence has emerged in support of the view that neural activity mediates mental processes by which we perceive, act, feel, and remember. The Neuron Theory supported by Ramon y Cajal (López-Muñoz et al., 2006), and Sherrington's identification of the synapse and vision of an integrative neural system (Levine, 2007), have flourished into an effortful search for the molecular substrates of human cognition and behavior. *In vivo* animal studies, and *in vitro* observations of cultured or *ex vivo* cells have identified multiple mechanisms...
implicated in sensation and behavior. This thesis focuses on glutamatergic receptors that have been established as mediators of excitatory transmission in the ACC. Through a combination of behavioral, pharmacological, biochemical, and electrophysiological methods, this thesis examined how behaviors observed in mouse models of fear learning, chronic pain, and itch correspond with in vitro observations of ACC neuronal activity. Data will be presented from three major projects published as separate manuscripts in peer reviewed journals that bridge in vivo observations with in vitro mechanisms highlighting how cortical synaptic activity within the ACC mediates fear learning and sensation related behaviors.

1.2 The anterior cingulate cortex

1.2.1 Introduction

The anterior cingulate cortex (ACC) has captivated research attention due to its involvement in multiple roles including emotion, executive function, physiological pain, social pain, and attention (Devinsky et al., 1995; Davis et al., 1997; Vogt, 2005; Zhuo, 2008). The ACC surrounds the frontal part of the corpus callosum, and consists of different layers of pyramidal cells and local interneurons, organized into layers I – III, and deeper layers V-VI, with a notable absence of layer IV(Vogt et al., 2005). Pyramidal cells are located in layers II, III, V, and VI, and they receive projections from the thalamus as well as other subcortical structures (Vogt, 2005; Lee et al., 2007b). The
ACC receives various direct and indirect inputs from various brain regions including the thalamus, amygdala and hippocampus (Wei et al., 1999; Vogt, 2005; Vogt et al., 2005; Zhuo, 2008), and also displays robust intracortical connections (Yang et al., 2006). Several observations have divided the ACC into distinct emotion and cognition related subareas (Bush et al., 2000). The emotion related area composes the more ventral part of the ACC, which receives robust projections from the amygdala (Vogt and Pandya, 1987) and projects to the periaqueductal grey (PAG), which has been implicated in modifying motor responses and plays a key role in defensive and emotional behaviors. The cognition related area is located in the dorsal part of the ACC, and is believed to be involved in mediating attention. Lesions in this area disrupt cognitive processes associated with motor events related to response selection (Devinsky et al. 1995). As we shall see in the following sections, such robust reciprocal connections with nociceptive and emotion related areas, in combination with strong intracortical connections, places the ACC in a great position to integrate various sources of information to mediate appropriate behavioral responses to external stimuli.

1.2.2 Nociceptive input to the ACC

Nociception is the ability to detect pain-producing (noxious) stimuli (Craig, 2003; Basbaum et al., 2009). The ACC is part of a specialized nociceptive neural system (Craig, 2003) that is involved in the integration of noxious stimuli with cognitive and emotional processing in the brain (Basbaum et al., 2009) (Figure 1.1). The skin contains
a variety of receptors, called nociceptors, which can respond to harmful stimuli such as mechanical stress, heat, cold, and noxious chemicals (Andrew and Craig, 2001; Craig, 2003). These receptors are found on primary sensory neurons, which transit information from the periphery to the spinal cord, where most terminate on lamina I neurons in the dorsal horn (Craig and Kniffki, 1985; Todd, 2010). These afferent fibres consist of the faster conducting, lightly myelinated, medium diameter Aδ-fibres, and the slower conducting, unmyelinated, small diameter C-fibres (Basbaum et al., 2009). The Aδ-fibres are believed to mediate acute sharp pain, whilst the C-fibres are understood to be responsible for delayed, more diffuse pain, referred to as first and second pain respectively (Basbaum et al., 2009). Most C-fibre neurons are polymodal, whereby they respond to both mechanical and thermal stimuli, although some C-fibres are activated only by heat (Basbaum et al., 2009). Most C-fibres are also responsive to noxious chemical stimuli (Craig, 2003; Basbaum et al., 2009), and to histamine (Andrew and Craig, 2001) and non-histaminergic pruritogens (itch inducing stimuli) (Davidson et al., 2007; Akiyama et al., 2009a). The existence of itch specific neurons is presently under debate, where some genetic mouse studies (Sun and Chen, 2007) and primate spinothalamic tract (STT) recordings (Andrew and Craig, 2001) provide some evidence for this hypothesis, whilst recordings from the thalamus of primates show that STT neurons can be activated by both pain and itch (Davidson et al., 2012). Mechanisms of itch sensation will be discussed in further detail in Chapter 4. The STT is the most prominent ascending nociceptive pathway in the spinal cord, and is made up of axons from nociceptive neurons in lamina I and from wide dynamic range neurons in laminae V-VIII. These neurons decussate (project to the contralateral side) and ascend in the
anterolateral white matter, terminating in the thalamus (Basbaum et al., 2009; Todd, 2010).

The thalamus is made up of multiple nuclei that send projections to multiple brain areas including the amygdala (LeDoux, 2000), somatosensory cortex (Craig, 2003) and ACC (Lee et al., 2007b). Projections to the ACC are particularly robust and diverse (Vogt, 2005; Vogt et al., 2005), and the ACC receives inputs from more thalamic nuclei than any other region in the cortex (Devinsky et al., 1995). Thalamic projections to the ACC innervate layer II/III pyramidal neurons, and there is strong evidence that thalamic-ACC evoked potentials arise from thalamic neuronal axons extending through layers V/VI of the ACC and into layers II/III (Lee et al., 2007a). The STT is a major ascending nociceptive pathway, and many STT neurons are excited by various noxious stimuli (Schouenborg, 1984; Basbaum et al., 2009), thus spinal nociceptive input to the ACC may arise from STT neurons via thalamic projections to the ACC. Accordingly, in vivo extracellular recordings from anesthetized mice showed evoked excitatory potentials (EPSPs) in the ACC in response to electrical stimulation of the hindpaw (Wei and Zhuo, 2001). More recently, intracellular recordings in anesthetized rabbits revealed that noxious stimulation of the sciatic nerve produces robust EPSPs in ACC pyramidal neurons (Shyu et al., 2010), and previous extracellular field potential recordings of anesthetized rats showed that such stimulation activated the ACC via the medial thalamus (Wang et al., 2006). Remarkably, some ACC neurons respond to noxious stimulation on any part of the body, as in vivo recordings from rabbits showed that a single ACC neuron can discharge in response to noxious stimulation on different parts of the body surface (Sikes and Vogt, 1992).
Human neuroimaging studies have shown that the ACC is active in correspondence with the presentation of noxious stimuli (Davis et al., 1997; Derbyshire et al., 1998; Lenz et al., 1998), pruritogens (Ikoma et al., 2006; Yosipovitch et al., 2008), threatening stimuli, (Marschner et al., 2008) (Alvarez et al., 2008) (Buchel et al., 1999), social rejection (Eisenberger et al., 2003), and imaginary pain (Craig et al., 1996). Electrophysiological recordings from ACC neurons have shown that they respond to noxious stimuli and in response to activity of nociceptive specific neurons (Hutchison et al., 1999; Wei and Zhuo, 2001). Moreover, human patients with cingulotomies report an attenuation of the unpleasantness that accompanies pain (Vadakkan et al., 2006), and animal studies have shown that lesions of the ACC result in a robust reduction of acute nociceptive responses (Johansen et al., 2001). More recently, single-unit recordings from the ACC in conscious rats showed increases in response frequencies corresponding to increases in noxious stimuli intensities (Kuo et al., 2009). Collectively these reports indicate that the ACC is active in correspondence with noxious input.

1.2.3 Affective and learning related input into the ACC

Human neuroimaging studies have shown that the ACC is also activated by fear and emotionally salient stimuli (Bishop et al., 2004; Vogt, 2005; Zhuo, 2008). Correspondingly, anatomical tracing studies have also identified projections to the ACC from other brain areas including robust input from the amygdala (Devinsky et al., 1995; Vogt et al., 2005; Bissiere et al., 2008; Shyu et al., 2010) hippocampus (Vogt and Pandya, 1987), and cortical regions (Zhuo, 2008). The amygdala is a brain structure...
located deep in the medial temporal lobe which has been repeatedly shown to play a critical role in emotional processing (LeDoux, 2000), and has been observed to play a key role in fear learning (Rogan et al., 1997; Schafe et al., 2001). Projections from the amygdala thus may allow the ACC to receive input regarding emotional valence of an event. Indeed, human neuroimaging studies have shown that threatening stimuli correspond with activation of the amygdala and the ACC (Bush et al., 2000), and that visual attention to threat can be modulated via an amygdala-ACC network (Carlson et al., 2012). Interestingly, a recent human neuroimaging study showed that an ACC region that borders the emotion-cognition subdivisions of the ACC is involved in attention to emotionally relevant stimuli (Carlson et al., 2012). Specifically, they observed a significant positive correlation between gray matter volume in this region and attention to threatening stimuli, suggesting that this ACC area is important for emotion–cognition interactions. Remarkably, although the amygdala is activated by threatening stimuli, gray matter volume in the amygdala did not correlate with attention bias to threat. One possibility is that the amygdala is critical for the initial recognition of threat and relays this information to the ACC to facilitate attentional and perceptual processing.

The combination of amygdala and thalamic input to the ACC, in addition to its robust intracortical connections, place the ACC in a position to integrate various nociceptive and emotional stimuli, suggesting a possible role for the ACC in pain affect. Several observations support this notion, and human neuroimaging studies have indicated that the ACC mediates the emotional component of pain (Rainville et al., 1997; Price, 2000). A set of studies used a clever approach to dissociate the affective
and sensory components of pain perception by combining hypnotic suggestion and positron emission tomography (PET). It was observed that increases in ACC activity were positively correlated with increases in the unpleasantness, but not in the intensity, of pain (Rainville et al., 1999). Notably such correlations were absent in the somatosensory cortex, highlighting a role for the ACC in pain affect, and perhaps a reflection of the strong connections between the ACC and emotional areas. Similarly, chronic pain patients with lesions in the ACC report reductions in the unpleasantness of pain (Price, 2000). Animal studies also support a role for the ACC in the affective component of pain. In rats for example, pharmacological glutamatergic stimulation in the ACC is sufficient to produce aversive conditioning in a condition place avoidance paradigm (Johansen and Fields, 2004), and electrical stimulation of the ACC paired with an auditory cue is sufficient to induce conditioned freezing in mice (Tang et al., 2005).
Figure 1.1: Diagram showing nociceptive transmission pathways in the central nervous system. Under normal physiological conditions, noxious stimuli activate nociceptive afferent Aδ and C-fibres. Incoming action potentials trigger a release of excitatory transmitter glutamate in the spinal dorsal horn (DH). In addition, some neuropeptides are also released, including substance P and neurokinin A. Glutamate and neuropeptides activate spinal dorsal horn neurons that decussate and ascend up the spinothalamic tract (STT). Thalamic neuronal projections relay nociceptive signals to major brain areas, including ACC, S1, S2, amygdala, and hippocampus. These brain
areas are in turn activated, and contribute to different aspects of pain perception, including the unpleasantness of pain. DRG = dorsal root ganglion.

1.2.4 Efferent projections from the ACC

The ACC projects out to multiple brain regions that allow it to modulate behavioral responses to noxious and emotional stimuli (Devinsky et al., 1995). Retrograde and anterograde labelling studies have found ACC projections to the PAG in monkeys (Muller-Preuss and Jurgens, 1976) and rats (Floyd et al., 2000). The PAG and rostral ventral medulla (RVM) are part of the endogenous analgesia system, whereby activation of PAG or RVM neurons decreases noxious stimuli-induced dorsal horn activity and nociception induced spinal reflexes (Calejesan et al., 2000). Remarkably, electrical or chemical stimulation of ACC neurons facilitates spinal reflexes, an effect that is reversibly blocked by lidocaine injection into the RVM (Calejesan et al., 2000), indicating that the ACC can modulate spinal nociceptive responses. Another prominent role of the PAG is in mediating emotional behaviors associated with threatening events (Devinsky et al., 1995), suggesting a possible pathway through which the ACC may modulate behavioral responses to fearful events. The ACC also sends projections to spinal and cortical motor areas. Observations employing retrograde tracers injected into the primary (M1) and supplementary (M2) motor areas of rhesus monkeys (Morecraft and Van Hoesen, 1992) revealed strong inputs from the ACC into these areas, with a notable larger representation in M2. Robust projections have also been observed to all
major components of the striatum (Devinsky et al., 1995). Most of the ACC neurons that project to motor systems are located in layer V (Devinsky et al., 1995). More recently, a study with monkeys showed that injection of a transsynaptic virus into the cervical spinal cord labelled neurons in the thalamus and ACC, and revealed some projections back to the spinal cord, the primary motor cortex and premotor areas (Dum et al., 2009), suggesting that the ACC can integrate nociceptive input and modulate motor output.

1.3 Excitatory transmission in ACC

1.3.1 Introduction to excitatory transmission in ACC

Glutamate is the major fast excitatory neurotransmitter within the ACC (Sah and Nicoll, 1991; Zhuo, 2008). Multiple ionotropic glutamate receptors have been observed to be activated by glutamate in the ACC, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Sah and Nicoll, 1991; Xu et al., 2008), kainate (KA) (Wu et al., 2005a), and N-methyl-D-aspartate (NMDA) (Wei et al., 2001; Zhao et al., 2005) receptors. AMPA and KA receptors mainly mediate rapid responses to glutamate (Sah and Nicoll, 1991), whilst NMDA receptors mediate a slower phase of the postsynaptic response and have been implicated in synaptic plasticity (Song and Huganir, 2002). A class of G-protein coupled receptors, the metabotropic glutamate receptors (mGluRs), also mediate slow glutamatergic transmission in the ACC but will
not be discussed in this thesis. Glutamatergic transmission in the ACC has been investigated both in vitro and in vivo, and many of the cellular and molecular mechanisms of synaptic transmission within the ACC have been identified. As we shall see in subsequent sections, the behavioral correlates of these mechanisms are also beginning to emerge.

1.3.2 AMPA receptors

AMPA receptors are ligand-gated, transmembrane, cation permeable channels that are located on the postsynaptic membrane, where they mediate the postsynaptic response to presynaptic glutamate release (Malinow and Malenka, 2002; Shepherd and Huganir, 2007). AMPA receptors are proteins assembled from a combination of four subunits, GluA1-GluA4 (previously GluR1-GluR4), which form hetero-tetramers most often composed of GluA1/GluA2 or GluA2/GluA3 combinations (Rosenmund et al., 1998). The different AMPA receptor subunits display similar extracellular and transmembrane regions, but have distinct intracellular cytoplasmic tails and display distinct functional properties (Hollmann and Heinemann, 1994). Alternative splicing of the C-terminal domains determines interactions with specific cytoplasmic proteins (Shepherd and Huganir, 2007), and provide the binding sties for protein phosphorylation (Roche et al., 1996; Malinow and Malenka, 2002). AMPA receptors have inwardly rectifying current/voltage (I/V) relations except in heteromeric complexes containing the GluA2 subunit, which display outward rectification (Hollmann and Heinemann, 1994).
RNA editing of a glutamine to an arginine residue in the ion channel pore region of the GluA2 subunit determines the characteristic outward rectification and makes the channel impermeable to Ca\(^{2+}\) (Hollmann and Heinemann, 1994; Shepherd and Huganir, 2007). In contrast, GluA2-lacking AMPA receptors are permeable to Ca\(^{2+}\), and display inward rectification.

Within the ACC, postsynaptic AMPA receptors mediate the majority of rapid excitatory postsynaptic responses (Sah and Nicoll, 1991; Wei et al., 1999; Zhuo, 2008). In vitro recordings from layer II/III ACC pyramidal neurons showed that excitatory postsynaptic currents (EPSCs) can be evoked by stimulation of layer V (Wei et al., 1999), which were completely blocked by bath application of the AMPA and KA receptor antagonist CNQX.

1.3.3 Activity dependent AMPA receptor recruitment

Synaptic excitatory transmission in the CNS, is plastic, and can undergo long term enhancement or attenuation, termed long term potentiation (LTP) and long term depression (LTD), respectively (Bliss and Collingridge, 1993; Malenka and Bear, 2004). LTP can be induced experimentally in electrophysiological in vitro brain slice recordings, and is understood to be the mechanism that drives memory storage within the brain (Bliss and Collingridge, 1993; Kandel, 2001b). Potentiation of synaptic excitatory transmission can be mediated through presynaptic alterations, such as enhanced neurotransmitter release, or through postsynaptic changes, such as recruitment of postsynaptic receptors. Presynaptic forms of LTP appear to be mediated by increases
in intracellular Ca$^{2+}$ levels and subsequent alterations of neurotransmitter release (for a review see Nicoll and Malenka, 1995); although this form of LTP clearly depicts an important mechanism for synaptic potentiation, it has yet to be observed in the cortex and thus will not be further discussed in this thesis. Postsynaptic mechanisms of LTP have received the most attention, and have been investigated for decades. Most investigations of LTP have been performed in hippocampal circuits, and thus most of the known mechanisms have been observed in hippocampal structures. It is therefore important to note that many of the mechanisms have yet to be fully confirmed in the cortex. A key component of postsynaptic LTP expression is the upregulation of postsynaptic AMPA receptors (Song and Huganir, 2002), and LTP is absent in the CA1 region of the hippocampus of adult GluA1 knockout mice (Zamanillo et al., 1999), indicating that GluA1 subunits are critical for LTP. Under normal basal conditions, most AMPA receptors in the CNS contain the GluA2 subunit, and very few GluA1 subunits are expressed on pyramidal neurons. In response to neuronal activity however, GluA1 subunits are recruited (Song and Huganir, 2002). In an elegant display of this phenomenon, GFP tagged GluA1 subunits were expressed in cultured hippocampal neurons, and were visualized using two-photon microscopy whilst being exposed to high frequency tetanic stimulation (Shi et al., 1999). Remarkably, whereas AMPA receptor GluA1 subunits were mostly intracellular prior to stimulation, tetanic synaptic activity induced a rapid insertion of GFP-GluA1 and clustering at dendrites; an effect that is mediated by the GluA1 carboxyl terminus (Shi et al., 2001). In contrast, subsequent observations of GFP tagged GluA2 subunits showed they continuously replace existing
synaptic receptors in a manner not requiring activity and mediated by the GluA2 carboxyl terminus (Shi et al., 2001).

GluA1 subunits are regulated by protein phosphorylation at serine 831 and 845 on its intracellular C-terminus domain, and LTP induction increases phosphorylation of GluA1 subunits (Lee et al., 2000). Accordingly, protein kinase A, an enzyme that is stimulated by cyclic AMP (cAMP) in an activity dependent manner, phosphorylates GluA1 subunits at their serine 845 site, and PKA application enhances glutamate gated currents by 40% in GluA1-HEK cells (Roche et al., 2006). Furthermore, disruption of the PDZ-ligand sequence of the GluA1 C-terminus blocks activity dependent GluA1 delivery (Song and Huganir, 2002), suggesting that PDZ-domain proteins are involved in dendritic delivery of GluA1. Importantly, activity dependent upregulation of GluA2 lacking, GluA1 containing Ca^{2+}-permeable AMPA receptors (CP-AMPARs) has been shown to be critical for early stages of long term enhancements in synaptic transmission in learning and emotional related brain areas. In the CA1 region of the hippocampus for example, LTP induction through theta burst stimulation and pairing protocols induced transient increases of CP-AMPARs (Plant et al., 2006; Guire et al., 2008).

1.3.4 AMPA receptors and potentiation of excitatory transmission at ACC synapses

Although LTP has been mostly investigated in the hippocampus, it has also been observed in the ACC, where it can be mediated by post-synaptic mechanisms that require AMPA receptor activity for induction. Much like in the hippocampus, GluA1 subunits in ACC neurons appear to be recruited by synaptic activity, and studies
combining genetic and pharmacological approaches have shown evidence that the AMPA receptor GluA1 subunit plays a critical role in ACC LTP. For example, application of a GluA1 inhibiting peptide (Pep1-TGL) prior to initiation of LTP induction protocols completely blocked LTP induction by paired training (Toyoda et al., 2007a), whereas there were no observable effects on LTP induction if Pep1-TGL was introduced 5 min after pairing protocol commenced. These findings indicate that functional recruitment of AMPA receptor GluA1 subunits is necessary for ACC LTP and is complete within 5–10 min. Importantly, the GluA1 inhibiting peptide Pep1-TGL selectively binds to its C-terminus, suggesting that ACC LTP requires the interaction between the C-terminus of GluA1 subunits and PDZ domain proteins in the postsynaptic membrane (Toyoda et al., 2007a). Correspondingly, pharmacological experiments showed that blockade of GluA2-lacking, CP-AMPARs through the application of Philanthotoxin-433 (PhTx) 5 min after paired training attenuates synaptic potentiation, whereas PhTx had no effect on basal responses (Toyoda et al., 2007a). These findings indicate that activity induced LTP in the ACC requires CP-AMPARs. In contrast, the AMPA receptor GluA2/3 subunits may help maintain constant synaptic transmission in an activity-independent manner (Toyoda et al., 2009b). Interestingly, disrupting interactions between AMPA receptor GluA2/3 subunits and PDZ domains had no effect on ACC LTP; although effects were observed in ACC LTD (Toyoda et al., 2007b), suggesting different roles for AMPA receptor GluA1 and GluA2/3 subunits in ACC LTP and LTD respectively. These findings were further supported by data from genetic mutant mice, whereby brain slice recordings from GluA1 knockout mice showed that ACC LTP was completely abolished, whereas ACC LTP was unaffected in mice lacking GluA2 (Toyoda et al., 2009b).
1.3.5 NMDA receptors

Glutamate is also the major ligand for a class of ionotropic channels highly permeable to Ca\(^{2+}\), the NMDA receptors (Hollmann and Heinemann, 1994). These ligand-gated, transmembrane channels are heteromers composed of GluN1 (previously NR1), GluN2A-D (previously NR2A-D), and GluN3 (previously NR3) subunits (Hollmann and Heinemann, 1994; Collingridge et al., 2009). Functional NMDA receptors are heteromers that require expression of at least one GluN1 and one or more of the GluN2A-D subunits, and are 5-10 times more permeable to Ca\(^{2+}\) than AMPA receptors (Malenka and Bear, 2004). In addition to requiring binding of presynaptically released glutamate, NMDA receptors are voltage dependent, displaying an extracellular Mg\(^{2+}\) block that is released when the postsynaptic membrane is depolarized (Malenka et al., 1999). Several in vitro observations in the ACC have detected NMDA receptor-mediated slow synaptic responses (Sah and Nicoll, 1991; Liauw et al., 2003; Liauw et al., 2005). More recently, electrophysiological recordings with intact thalamic-ACC afferent showed that thalamic evoked ACC potentials where partially blocked by the NMDA receptor antagonist AP-5, indicating that NMDA receptors mediate a component of the evoked postsynaptic response (Lee et al., 2007b). Importantly, in vivo recordings from freely moving mice revealed that NMDA receptors contribute to slow EPSP responses in the ACC (Wu et al., 2005a), highlighting an important correlation between in vitro and in vivo observations. NMDA receptor activity however requires depolarization of the postsynaptic membrane potential, and thus CNQX can completely abolish excitatory
currents in the ACC without the presence of AP-5 (Wei et al., 1999). This indicates that whereas under normal physiological conditions most synaptic responses within the ACC are carried out by AMPA receptors, NMDA receptors function in an activity-dependent manner.

NMDA receptors are expressed at the postsynaptic membrane, as well as extrasynaptically (Malenka et al., 1999). NMDA receptor GluN2A and GluN2B subunits are the most prominently expressed in forebrain structures (Sah and Nicoll, 1991), and NMDA receptor mediated currents within the ACC are mostly driven by receptors containing GluN2A or GluN2B subunits, accounting for ~70 % and ~18 % of NMDA mediated currents respectively (Zhao et al., 2005). Bath application of the GluN2A antagonist, NVP-AAM077, in combination with the GluN2B antagonists, ifenprodil or Ro25-6981, can almost completely block NMDA receptor mediated EPSCs.

1.3.6 NMDA receptors and ACC synaptic plasticity

NMDA receptors are considered coincidence detectors as they require binding of glutamate to the receptor concurrently with depolarization of the postsynaptic membrane (Malenka et al., 1999). NMDA receptor activation induces rapid AMPA receptor insertion in hippocampal neurons (Shi et al., 1999; Lu et al., 2001; Pickard et al., 2001), and induces LTP of excitatory transmission (Lu et al., 2001). Similarly, a major form of LTP in the ACC requires activation of postsynaptic NMDA receptors. Activation of postsynaptic NMDA receptors and L-type voltage gated calcium channels (L-VDCCs) induces increases of intracellular Ca$^{2+}$, that can trigger intracellular signaling
cascades involved in LTP induction (Huang and Malenka, 1993; Malinow and Malenka, 2002). Thus as NMDA receptors are calcium permeable, and require postsynaptic depolarization for activation, they can be responsible for activity dependent Ca^{2+} influx. Increases in intracellular Ca^{2+} in turn are necessary for activity dependent changes in synaptic transmission (Malinow and Malenka, 2002). Indeed, the Ca^{2+} chelator BAPTA blocks Ca^{2+} activated currents, and completely prevents LTP induction in the ACC, highlighting the necessity of elevated postsynaptic Ca^{2+} concentrations for the induction of ACC LTP (Zhao et al., 2005). Accordingly, multiple protocols that can induce LTP in ACC synapses are sensitive to the inhibition of NMDA receptors (Zhao et al., 2005). For example, ACC slice recording experiments demonstrated that bath application of the NMDA receptor antagonist, AP-5, blocks LTP induction by TBS (Liauw et al., 2005). Interestingly, the NMDA receptor GluN2A and GluN2B subunits have both emerged as important modulators of ACC LTP, as bath applications of either GluN2A or GluN2B selective antagonists will reduce LTP, and co-application of both antagonists completely blocks it (Zhao, 2005).

1.3.7 NMDA mediated potentiation and gene expression

Long term changes in synaptic transmission are often mediated via activity-dependent changes in gene expression (Vignes and Collingridge, 1997; Kandel, 2001b), and long term expression of LTP is sensitive to protein transcription and protein translation inhibitors (Kandel, 2001). NMDA receptor mediated increases of intracellular Ca^{2+} can trigger signaling cascades including cAMP and Ca^{2+} calmodulin dependent
protein kinase pathways (Bourtchuladze et al., 1994) (Figure 1.2). These pathways in turn lead to the phosphorylation of the transcription factor cAMP responsive element binding protein (CREB) at the serine 133 site; this phosphorylation is critical for activation of CREB (Sheng et al., 1991). Activation of CREB promotes cAMP-responsive element (CRE) dependent transcription, and has been implicated in expression of a number of genes, including immediate early gene (IEG) like c-fos (Hunter and Karin, 1992). In hippocampal cultures, synaptic stimulation-induced intracellular Ca\(^{2+}\) influx caused rapid CREB phosphorylation (Deisseroth et al., 1996).

Interestingly, although this study observed that CREB phosphorylation was initiated by increases in Ca\(^{2+}\) concentrations near the cell membrane, it also observed phosphorylation even when elevation in bulk internal Ca\(^{2+}\) was suppressed with internal EGTA, implicating second messenger systems linking Ca\(^{2+}\) influx with CREB activation. Indeed, in subsequent investigations, this same group performed immunoreactivity studies on cultured hippocampal neurons and showed that brief bursts of synaptic stimulation rapidly increased Ca\(^{2+}\)/calmodulin-dependent protein kinase IV (CaMKIV) immunoreactivity in the nucleus, leading to the conclusion that influx of Ca\(^{2+}\) triggers nuclear CREB phosphorylation via CaMKIV (Deisseroth et al., 1998).

In the ACC, CREB is also activated in an activity-dependent manner, is downstream from CaMKIV, and is believed to mediate long term enhancements in excitatory transmission (Wei et al., 2002b; Wei et al., 2002a). For example, genetic deletion of CaMKIV prevents TBS-induced synaptic potentiation in the ACC (Wei et al., 2002a), whereas transgenic mice with overexpressed CaMKIV display enhanced ACC LTP (Wu et al., 2008). Interestingly, early potentiation is also enhanced, suggesting a
possible early contribution of CREB to synaptic potentiation in the ACC. In accordance with the belief that LTP mediates learning, and that long term LTP requires CREB mediated gene transcription, activation of CREB has been implicated in formation of long-term memory (Bourtchuladze et al., 1994; Silva et al., 1998; Kida et al., 2002).

**Figure 1.2:** Activity dependent CREB-transcription mediates AMPAR upregulation during LTP. Synaptic activity induces Ca^{2+} influx via NMDARs and initiates AMPA receptor (AMPA) upregulation through activation of Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) related pathways. Intracellular Ca^{2+} binds to calmodulin.
(CaM) and leads to the activation of adenyl cyclases (ACs), including AC1, and Ca$^{2+}$/CaM-dependent protein kinases, including PKA, PKC, and CaMKIV. These pathways lead to the phosphorylation of the transcription factor CREB, activating CRE mediated transcription.

1.3.8 Kainate receptors

KA receptors are tetrameric channels composed of a combination of five subunits: GluK1-GluK3 (previously GluR5–7) and GluK4-GluK5 (previously known as KA1 and KA2) (Collingridge et al., 2009). Selective AMPA receptor antagonists, and the more recent advancements in KA receptor antagonists, have revealed interesting details of KA receptor function in the CNS (Jane et al., 2009). KA receptor mediated currents have longer activation latencies and display slower decay rates than AMPA receptors (Castillo et al., 1997). Remarkably, KA receptors are located on both sides of the synapse (Lerma, 2006), where postsynaptic KA receptors have been observed to contribute to the excitatory postsynaptic current (Castillo et al., 1997; Vignes and Collingridge, 1997; Wu et al., 2005a), whilst presynaptic KA receptors appear to modulate synaptic transmission by regulating transmitter release (Chittajallu et al., 1996; Rodriguez-Moreno et al., 1997; Lerma, 2003, 2006).

1.3.9 Postsynaptic KA receptors
In the CA3 region of the hippocampus, activation of postsynaptic KA receptors appears to require repetitive synaptic activity. In guinea pigs, KA receptor mediated synaptic potentials were measured by recording whole cell EPSPs in the presence of the selective AMPA receptor antagonist, GYKI 53655 (Castillo et al., 1997). Whereas fast EPSPs evoked by single-shock mossy fibre stimulation were completely blocked by GYKI 53655, repetitive stimulation yielded a slow component that emerged in the continued presence of GYKI 53655, but was blocked by CNQX, indicating that postsynaptic KA receptors are activated by repetitive activity at CA3 synapses. Similar observations were made in rat hippocampal CA3 slices, where single-shock stimulation failed to evoke any postsynaptic KA current, but an obvious response was detectable following high frequency stimulation (Vignes and Collingridge, 1997).

In contrast to the hippocampus, postsynaptic KA receptor activity at ACC synapses can be induced by single-shock stimulation. Specifically, small KA receptor-mediated EPSCs were observed in the presence of the NMDA receptor antagonist, AP-5, and the selective AMPA receptor antagonist, GYKI 53655 (Wu et al., 2005a). High-frequency repetitive stimulation however was able to significantly facilitate KA mediated EPSCs, suggesting that activity can recruit additional KA receptors. Furthermore, genetic deletion of the GluK1 subunit significantly reduces KA EPSCs and kainate activated currents in ACC pyramidal neurons (Wu et al., 2005a), indicating that GluK1-containing KA receptors play a prominent role in KA mediated ACC synaptic transmission.
1.3.10 Presynaptic KA receptors

Presynaptic KA receptors exhibit biphasic regulation of transmitter release. In one of the earliest investigations employing specific AMPA receptor antagonists to study KA receptor mediated activity in the CA1 region of the hippocampus, kainate induced KA receptor activation resulted in a brief enhancement of postsynaptic currents followed by long lasting depression (Chittajallu et al., 1996). The presynaptic location for this form of depression was later confirmed in CA3-CA1 hippocampal synapses, where combined methods, including microdissection of the somatodendritic portions of CA3 fibres and selective AMPA receptor antagonists, confirmed the EPSC attenuating effects of presynaptic KA receptor activity (Frerking et al., 2001). In the same year, this group also revealed that KA receptors can also mediate a presynaptic mechanism of transmission enhancement (Schmitz et al., 2001). Specifically, kainate application at levels much lower than were previously seen to depress transmission (50 nM vs. 1 µM) was observed to induce a rapid enhancement of EPSCs. Remarkably, these patterns were replicated by synaptic activity whereby brief tetanic stimulation enhanced mossy fibre responses, but prolonged tetanus had an attenuating effect. Thus, whereas mild stimulation promotes KA mediated transmitter release, stronger stimulation induces KA mediated inhibition of release. Presynaptic KA mediated depression of CA1 and CA3 pyramidal neurons has also been observed in response to the selective and potent GluK1 agonist, ATPA, suggesting that GluK1 containing KA receptors play a prominent role in KA mediated presynaptic activity (Jane et al., 2009).
1.3.11 Modulation of inhibitory transmission

Presynaptic KA receptors also control the release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA); binding of GABA at postsynaptic GABA<sub>A</sub> receptors produces inhibitory postsynaptic currents (IPSCs), decreasing the likelihood of action potential generation in the postsynaptic cell. Neuronal recordings from cultured hippocampal neurons and CA1 pyramidal cells showed that bath application of kainate or glutamate, (in the presence of AMPA, NMDA, and mGluR receptor blockers), decreased the amplitudes of evoked IPSCs by up to 90%, indicating a robust role in KA mediated evoked inhibitory transmission (Rodriguez-Moreno et al., 1997). This form of dis-inhibition was dose dependent, and of presynaptic origin, displaying an attenuating effect on spontaneous presynaptic GABA release. Remarkably, ATPA induced similar reductions in IPSCs (up to 77%) in primary rat cultured neurons, indicating that GluK1 containing KA receptors are important modulators of presynaptic GABA release (Clarke et al., 1997). More recently, observations in the amygdala have provided further evidence that presynaptic GluK1 subunits exert bidirectional control on GABA release (Braga et al., 2003). Lower concentrations of ATPA (300 nM) enhanced the frequencies of miniature IPSCs (mIPSCs), whilst larger concentrations (10 μM) had a suppressing effect. These effects were blocked by a GABA antagonist, bicuculin, confirming they were GABAergic currents, and were also prevented if a selective GluK1 antagonist was included in the bath solution. These effects were observed to be of presynaptic origin, as ATPA did not induce an effect on the amplitude of mIPSCs. A change in frequency but not amplitude of mIPSCs indicates changes in the probability of quantal release of
transmitter at presynaptic terminals; although KA receptors located at somatodendritic regions can induce GABA release in the hippocampus (Frerking et al., 1998) and amygdala (Braga et al., 2003). An ATPA mediated decrease in presynaptic GABA release has also been observed in pyramidal cells of the rat motor (Ali et al., 2001) and prefrontal (Mathew et al., 2008) cortices. In the ACC, bath application of low concentrations of ATPA (3 µM) significantly increased evoked IPSCs, whereas higher concentrations (10 µM) attenuated IPSCs (Wu et al., 2007). Interestingly, ATPA induced effects on evoked currents were sensitive to blockade of voltage gated Ca	extsuperscript{2+} channels, and had no effect on mIPSCs, suggesting that ATPA mediated effects via GluK1 containing KA receptors on somatodendritic regions of ACC interneurons.

Table 1: ACC ionotropic glutamate receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Activation</th>
<th>Kinetics</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>AMPA</td>
<td>Ligand gated</td>
<td>Fast</td>
<td>Postsynaptic - GluA1 recruitment critical for LTP</td>
</tr>
<tr>
<td>NMDA</td>
<td>Ligand gated &amp; voltage dependent Mg	extsuperscript{2+} block release</td>
<td>Slow</td>
<td>Postsynaptic - GluN2A, GluN2B mediate intracellular events for LTP</td>
</tr>
<tr>
<td>Kainate</td>
<td>Ligand gated</td>
<td>Fast and slow</td>
<td>Presynaptic - transmitter release</td>
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<tr>
<td></td>
<td></td>
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<td>Postsynaptic - contribution to excitatory current</td>
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Figure 1.3: Diagram showing ACC excitatory synapse undergoing LTP. Activity dependent presynaptic glutamate (Glu) release activates postsynaptic AMPA and KA receptors. Repeatetive activation depolarizes the postsynaptic membrane and activates NMDA receptors, resulting in an increase of intracellular Ca$^{2+}$, triggering cAMP and Ca$^{2+}$ calmodulin dependent pathways. In turn, Ca$^{2+}$ dependent protein kinases phosphorylate
glutamate AMPA receptors, increasing their sensitivity to extracellular glutamate and may lead to trafficking or insertion of additional AMPA GluA1 receptors. Presynaptic enhancements of excitatory neurotransmitter release can also potentiate the postsynaptic response although in vitro evidence of this in the ACC has yet to be determined.

1.4 Comments

Areas of the ACC are involved in affect and cognition, receiving projections from emotional and nociceptive areas respectively. The ACC also presents robust intracortical connections, and it has been proposed that the ACC integrates affective and cognitive components of external stimuli in order to execute appropriate behavioral responses. If the ACC integrates emotional and cognitive information about external stimuli in order to mediate behavioral responses, then neural activity within the ACC must correlate with behavioral output. This thesis focuses on glutamatergic receptors that have been established as mediators of excitatory transmission in the ACC. Do in vitro ACC observations reflect physiological mechanisms that are responsible for behavior? Through a combination of behavioral, pharmacological, biochemical, and electrophysiological methods, the following chapters will address if 1) LTP-like mechanisms within the ACC mediate pain induced trace fear learning; 2) If enhancements of activity dependent gene transcription in the ACC potentiate chronic pain behaviors; and 3) If pruritogen-induced behaviors are modulated by synaptic transmission in the ACC.
CHAPTER 2

RAPID SYNAPTIC POTENTIATION WITHIN THE ANTERIOR CINGULATE CORTEX MEDIATES TRACE FEAR LEARNING
CHAPTER 2: RAPID SYNAPTIC POTENTIATION WITHIN THE ANTERIOR CINGULATE CORTEX MEDIATES TRACE FEAR LEARNING

2.1 Background

Attention plays a critical role in modulating our experience of external stimuli. The ACC is involved in attention to threat, and receives inputs from nociceptive and emotion related brain areas. Potentiation of synaptic activity is believed to be the cellular substrate for learning and memory, and the ACC displays robust in vitro synaptic potentiation that is modulated by NMDA receptors, and requires AMPA receptor GluA1 subunits. Although the cortex has been extensively studied in the long-term memory storage of pain induced fear memory, less emphasis has been placed on immediate cortical contributions to fear memory formation. This chapter will cover literature indicating that trace fear learning engages the ACC, and will present evidence that immediate plasticity related events within the ACC are necessary for trace fear memory formation.
2.1.1 Mechanisms of fear learning

Associative fear conditioning pairs an arbitrary conditioning stimulus (CS), such as an auditory tone, with a noxious one (US), such as a foot shock. This form of conditioning results in robust freezing behavior in response to presentation of the CS in the absence of the US. Freezing is a defensive behavior that is exhibited by rodents when faced with predators (Fanselow and Poulos, 2005), and likely involves brain areas related to threat. Fear conditioning-induced changes in defensive behavior are believed to be a result of changes in neuronal activity in emotion related brain areas (LeDoux, 2000). It is well accepted that the cerebral cortex is involved in long term storage of memory, and LTP of central synapses is believed to be the basic mechanism that drives memory storage within the brain (Bliss and Collingridge, 1993; Kandel, 2001a). Indeed, LTP-like mechanisms have been observed in the amygdala in correspondence with fear conditioning (Rodrigues et al., 2004). For example, fear conditioning has been shown to induce enhancements in excitatory glutamatergic transmission in the amygdala (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Rumpel et al., 2005; Clem and Huganir, 2010). Accordingly, lesions of the amygdala interfere with contextual and auditory fear conditioning (Phillips and LeDoux, 1992), and lesions of the dorsal hippocampus block the acquisition and expression of contextual fear memory (Kim and Fanselow, 1992; Phillips and LeDoux, 1992).

Formation of memories initially relies on medial temporal lobe structures, including the hippocampus and amygdala, but over time become increasingly dependent on the cortex, eventually allowing memories to become independent of the
hippocampus (Bontempi et al., 1999; LeDoux, 2000; Frankland and Bontempi, 2005).
Indeed, long term stabilization of fear memory corresponds with time dependent
increases in dendritic spines in the ACC (Restivo et al., 2009), and cingulate neuronal
activity is not critical for recent (24 h) contextual fear memory recall (Frankland et al.,
2004). Nevertheless, ACC activity is critical for remote contextual fear memory (36
days) (Frankland et al., 2004), suggesting long term storage of fear memory occurs
within the ACC. The time dependent changes observed in the ACC during
consolidation of long term fear memory likely require mechanisms during acquisition to
‘tag’ the ACC for subsequent changes, thus allowing for future transfer of
information/memory to this region (Frey and Morris, 1997). Little is known however
regarding immediate cortical contributions to fear memory formation.

2.1.2 Fear learning involves the ACC

The ACC receives robust nociceptive input and receives multiple projections from
the amygdala (reviewed in Chapter 1), placing the ACC in a great position to integrate
important components of pain induced emotional learning. Neuroimaging studies show
that trace fear conditioning, which introduces a time interval between the CS and the
US, activates several brain regions in humans, including the amygdala, hippocampus,
medial prefrontal cortex (mPFC), and the ACC (Buchel et al., 1999; Knight et al., 2004).
ACC activity can also be detected when subjects focus their attention to threat related
stimuli (Bishop et al., 2004), and it is likely that attention plays a critical role in
modulating our experience of external stimuli. In accordance, animal studies have
confirmed that the ACC is engaged during trace fear conditioning, an associate fear conditioning paradigm that increases the attentional demand of the learning task by introducing a time interval between CS and US. For example, whereas trace fear conditioning was found to induce robust c-Fos expression in the ACC of rats, visual distractions between the CS-US pairings during conditioning blocked c-Fos expression and also prevented memory formation (Han et al., 2003).

2.1.3 Fear memory formation in the ACC: evidence for LTP-like mechanisms

Animal studies suggest that LTP-like mechanisms in the ACC can mediate fear learning. For example, contextual and auditory fear memory is enhanced in transgenic mice with forebrain overexpression of the NMDA receptor GluN2B subunit, which is an important modulator of ACC LTP in vitro (Tang et al., 1999). Moreover, immunostaining observations showed that ACC neurons express increases in CREB activation in response to trace fear learning (Wei et al., 2002a), and mice overexpressing CaMKIV show a pronounced improvement in trace fear memory that corresponds with enhancements in ACC LTP in layer II/III pyramidal neurons (Wu et al., 2008). It was later shown that CaMKIV is required for translation-dependent early synaptic potentiation within the ACC (Toyoda et al., 2010), further implicating ACC LTP related mechanisms in trace fear learning. More recently, electrophysiological recordings from freely moving mice show that ACC neurons are responsive to peripheral noxious stimuli and exhibit theta burst activity during trace fear learning (Steenland et al., 2012). These
data indicate that key mediators of *in vitro* LTP may also be responsible for *in vivo* trace fear learning in the ACC.

LTP induction in the ACC corresponds with recruitment of postsynaptic GluA1-containing CP-AMPARs (Toyoda et al., 2007a). Several studies indicate that CP-AMPARs mediate synaptic strengthening (Clem and Barth, 2006; Plant et al., 2006; Guire et al., 2008), and AMPA receptor plasticity is strongly implicated in learning and memory (Rumpel et al., 2005; Kessels and Malinow, 2009). In particular, in the CA1 region of the hippocampus, transient increases of CP-AMPARs were observed in response to LTP induction through theta burst stimulation (Guire et al., 2008) and pairing protocols (Plant et al., 2006). More recently, increases in CP-AMPARs were observed in the amygdala in response to auditory fear conditioning (Clem and Huganir, 2010). NMDA receptor activation can drive AMPA receptor insertion in the postsynaptic membrane in hippocampal pyramidal neurons *in vitro* (Shi et al., 1999), and GluN2B subunits in the hippocampus are necessary for trace fear memory formation (Huerta et al., 2000). Similarly, NMDA receptors are critical modulators of ACC LTP *in vitro*, and AMPA receptor GluA1 subunits are critical for ACC LTP. Although evidence suggests that the ACC is involved in trace fear learning, and remote expression of fear memory corresponds with ACC activity (Frankland et al., 2004), whether LTP-like events occur early within the cortex in response to trace fear learning, and whether it requires GluN2B subunits had not been previously investigated. The following results present data collected through integrative methods, including behavioral, pharmacological, biochemical, and electrophysiological, to determine if plasticity related events occur within the ACC during trace fear learning.
2.2 Methods

2.2.1 Animals

Experiments were performed with adult (8-12 week) male C57/BL6 mice purchased from Charles River (Quebec, Canada) or transgenic FosGFP mice obtained from the laboratory of Dr. Alison Barth (Carnegie Mellon University). Animals were housed under a 12h light/dark cycle, and all experiments were performed under protocols approved by the University of Toronto Animal Care Committee.

2.2.2 Fear conditioning

All conditioning was completed in an isolated shock chamber (Medical Associates, St Albans, VT, USA). Trace fear conditioning was performed as reported previously (Wu et al., 2008). The conditioned stimulus (CS) used was an 80-db white noise, delivered for 15 s, and the unconditioned stimulus (US) was a 0.75-mA electric foot-shock for 0.5 s. Mice were acclimated for 5 min, and were presented with 10 trials in the following order: CS – trace – US (trace period = 30 s, intertrial intervals = 210 s). For delay fear conditioning, the conditioning stimulus (CS) used was an 80-db white noise, delivered for 15 s, and the unconditioned stimulus (US) was a 0.75-mA electric foot-shock for 0.5 s that was presented at 14.5 sec into the CS presentation, such that
the CS and US co-terminated. For memory retrieval tests, mice were introduced to a novel chamber and were acclimated for 5 min and subjected to a presentation of the CS to test for trace fear memory (Huerta et al., 2000). All data were recorded using the video-based Freeze Frame fear conditioning system and analyzed by Actimetrics Software (Coulbourn Instruments, Wilmette). Average freezing for the baseline and for the trace period (30 s) following the CS during the training and testing sessions were analyzed. Freezing bouts (the absence of movement aside from respiration) lasting 1 s or more were considered as freezing.

2.2.3 ACC cannulae implantation and microinjection

We implanted bi-lateral cannulas into the ACC of mice as reported previously (Wu et al., 2005b). Mice were anaesthetized by intraperitoneal (IP) injections of a mixture of 1.3 mL of ketamine (100 mg/ml, Bimeda MTC, Cambridge, Ontario) and 0.5 ml of xylazine (20 mg/ml, Bayer, Toronto, Ontario, Canada) in 8.2 ml of normal saline at a dose of 10 µl per gram body weight. Mice heads were secured on a stereotaxic frame and 24-gauge guide cannulas were implanted bilaterally into the ACC (0.7 mm anterior to bregma, ± 0.3 mm lateral from the midline, 0.9 mm beneath the surface of the skull). Mice were given 2 weeks to recover after cannula implantation. Intra-ACC injections were delivered via a 30-gauge injection cannula that was lowered 0.85 mm further into the brain than the guide. The microinjection apparatus consisted of a Hamilton syringe (10 µl) connected to an injector needle (30 gauge) by a thin polyethylene tube and motorized syringe pump. All infusions consisted of 0.5 µl of solution delivered at a rate
of 0.05µl/min. Injection sites were confirmed at the end of all experiments and sites outside of the ACC region were excluded from the study.

### 2.2.4 Membrane preparation

Membrane preparation was performed as previously described (De March et al., 2008) with minor changes. ACC samples were dissected in cold D-PBS and resuspended in Buffer 1 (2 mM Tris–EDTA, 320 mM sucrose, 5 mM MgCl₂, and 1X protease inhibitor cocktail, pH 7.4), and homogenized. Each sample was centrifuged at 1000xg for 10 min and the supernatants (S1) were recovered. The remaining pellet (P1) was then resuspended in Buffer 2 (50 mM Tris–HCl, 2 mM Tris–EDTA, 5 mM MgCl₂, and 1X phosphatase inhibitor cocktail 1 and 2, pH 7.0) and centrifuged at 1000xg for 10 min, with its supernatant (S2) collected and combined with S1. The remaining pellet (P2) was resuspended in Buffer 2, and again centrifuged at 1000xg for 10 min., and its supernatant (S3) was combined with S1 and S2. Combined supernatant fractions (S1, S2 and S3) were finally centrifuged at 39,000xg for 30 min, the resulting supernatant contained the cytosolic fractions, and the resulting pellet (membrane fractions) was resuspended in Buffer 3 (50 mM Tris–HCl, 2 mM Tris–EDTA, 3 mM MgCl₂, and 1X phosphatase inhibitor cocktail 1 and 2, pH 7.4).

### 2.2.5 Western blot analysis
Western blot was performed as previously described (Wang et al., 2007). Sample protein concentrations were quantified using Bradford assay, and electrophoresis of equal amounts of protein was performed on NuPAGE 4-12% Bis-Tris Gels (Invitrogen, Carlsbad, CA). Separated proteins were transferred to polyvinylidene fluoride membranes (Pall Corporation, East Hills, NY) at 4°C overnight for analysis, and were then probed with primary antibodies as follows: anti-GluA1 (1:4000, rabbit polyclonal), anti-GAPDH (1:6000, mouse monoclonal), anti-GluA2/3 (1:1000, rabbit polyclonal), followed by horseradish peroxidase (HRP)-coupled secondary antibody diluted at 1:3000 for 2 hours followed by enhanced chemiluminescence detection of the proteins with Western lightning chemiluminescence reagent plus (PerkinElmer Life Sciences). ImageJ software (National Institute of Health) was used to assess the density of immunoblots by a blind observer.

2.2.6 Immunohistochemistry

Immunostaining was performed as described previously (Wu et al., 2008; Li et al., 2010). Mice were anesthetised with isoflurane and perfused with 0.01 mol/l phosphate-buffered saline (PBS; pH 7.4) via the ascending aorta followed by perfusion of 4% paraformaldehyde (PFA) in 0.1 mol/l PB at 4 °C. The brains were removed and post-fixed for 4 hours in 4% PFA, after which brains were placed in vials filled with 30% sucrose in 0.1 mol/l PB at 4 °C for at least 48 hours, or until the brain fully dropped to the bottom of the jar. Brain sections containing the ACC were cut using a cryostat (Leica) at 30 µm thickness. Briefly, sections were sequentially incubated through the
following solutions: (i) a solution of 3% bovine serum albumin (BSA; Sigma, St Louis, USA) and 0.3% Triton X-100 containing anti- c-Fos (1:500 abcam) primary antibody for 3 days at 4 °C. (ii) Biotin labelled goat anti-rabbit secondary antibody (1:1000 Santa Cruz, CA) for 24 hours at 4 °C (iii) Cy3 conjugated streptavidin (1:1000; Santa Cruz, CA, USA) for 2 hours at room temperature. In between each step, sections were rinsed with PBS 3 times for 10 min. Sections were mounted on gelatin coated slides, air-dried, cleared and cover-slipped for observation under a confocal microscope (FV-1000, Olympus, Japan).

2.2.7 Retrograde labelling

Retrograde labeling was performed as described previously (Ikeda et al., 2006b; Chen et al., 2008). Briefly, mice were anaesthetized with isoflurane (1–3%, as needed) and were placed in a stereotaxic apparatus. A hole was drilled through the skull in order to allow insertion of a 500-nl Hamilton syringe needle. The animals received a single injection of 200 nl of 2.5 % Dil into one side of the ACC, according to the mouse brain atlas (Paxinos and Franklin, 2004), through a sharp glass micropipette (tip outer diameters ranged from 10–20 μm) attached to a Hamilton syringe. After 3 days, mice were deeply anesthetized with isoflurane and perfused with 0.1 mol/l PB followed by 4% PFA in 0.1 mol/L PB as mentioned above. Coronal 30 μm thick ACC and amygdala slices were serially cut in a cryostat (Leica) and Dil injection sites in the ACC and
retrogradely labeled cells in the amygdala were then observed under a confocal microscope.

2.2.8 Electrophysiology

Coronal brain slices (300 µm) at the level of the ACC were prepared using standard methods (Wu et al., 2005b; Xu et al., 2008; Li et al., 2010) immediately after trace fear conditioning. Slices were transferred to a submerged recovery chamber with oxygenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgSO₄, 25 NaHCO₃, 1 NaH₂PO₄, and 10 glucose at room temperature for at least 1 hr. Experiments were performed in a recording chamber on the stage of a BX51W1 microscope equipped with infrared differential interference contrast optics for visualization. Excitatory post-synaptic currents (EPSCs) 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 of the ACC. AMPA/KΑ receptor-mediated EPSCs were induced by repetitive stimulations at 0.05 Hz, and neurons were voltage-clamped at -60 mV (without liquid junction potential correction) in the presence of AP5 (50 µM). The recording pipettes (3–5 MΩ) were filled with a solution containing (in mM) 124 K-gluconate, 5 NaCl, 1 MgCl₂, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na₃-GTP, and 10 phosphocreatine disodium (adjusted to pH 7.2 with KOH). Picrotoxin (100 µM) was always present to block γ-aminobutyric acid (A) (GABAₐ) receptor-mediated inhibitory synaptic currents in all experiments. The initial access resistance was 15–30 MΩ, and it
was monitored throughout the experiment. Data were discarded if the access resistance changed > 15% during an experiment. Data were filtered at 1 kHz, and digitized at 10 kHz. Electrophysiological recordings were performed by Drs. Xiang-yao Li, and Tao Chen.

2.2.9 Drugs

In order to block NMDA receptor GluN2B subunit activity, we used Ro-25-6981 or Ifenprodil (Tocris Bioscience), NMDA receptor antagonists that target the GluN2B subtype (Wu et al., 2005b; Fan et al., 2009). Mice were given i.p. injections of 10 mg/kg doses, and bilateral ACC 0.5µl infusions (2 µg/µl) for the microinjection studies. To block CP-AMPA receptors we used the antagonist, 1-naphthylacetyl spermine (NASPM).

2.2.10 Nociceptive behavioral tests

In the hotplate test, mice were placed on a standard thermal hotplate with a heated surface (55 °C) (Columbus Instruments, Columbus, OH). The latency for nociceptive responses was recorded with a cut-off time of 30 seconds. The spinal nociceptive tail-flick reflex was evoked by radiant heat (Columbus Instruments, Columbus, OH) applied to the underside of the tail, and latencies were measured with a cut-off time of 10 seconds.
2.2.11 Data Analysis

Results are expressed as mean ± SEM. Unpaired student t-tests and one-way Analyses of Variance (ANOVA) were performed. Significant effects were further tested by the Holm-Sidak test for multiple comparisons. In all cases $P < 0.05$ was considered statistically significant. Sample sizes were determined based on previous reports observing significant changes in membrane bound and whole sample AMPA receptor GluA1 expression in the ACC through Western blot (Xu et al., 2008); significant behavioral effects through systemic and intra-ACC pharmacological inhibition (Zhao et al., 2006; Wu et al., 2005); and significant changes in excitatory transmission in vitro (Xu et al., 2008).

2.3 Results

2.3.1 Evidence for ACC connections with the amygdala in adult mice

The ACC receives direct inputs from various nociceptive and emotional related areas including the thalamus and amygdala (Lee et al., 2007a; Vogt et al., 2008; Zhuo, 2008). These connections, along with observations that implicate its involvement in fear memory recall, suggest that the ACC may be part of a larger fear memory system that encompasses the hippocampus, amygdala, and ACC. To confirm amygdala-ACC
connections, injections of the retrograde tracer 1,1-dioactadecyl-3,3,3,3-tetramethylindocarbodyanineperchlorate (Dil) were performed into the ACC. These injections resulted in labelling of neurons within the basolateral and lateral amygdala in adult C57 mice (Figure 2.1 A-C). These results indicate that the neurons in the amygdala send direct projections to the ACC, confirming input to the ACC from key emotion areas known to be involved in auditory fear learning.

**Figure 2.1: The ACC forms connections with the amygdala.** (A) Injections of the retrograde tracer Dil were performed into the ACC. (B) Representative injection site. (C) Dil labelling of neurons within the amygdala in adult C57 mice; scale bar represents 20 µm.

2.3.2 Trace fear learning upregulates membrane AMPA receptor GluA1 subunits within the ACC
In order to investigate trace fear learning induced alterations, a conditioning paradigm was used that pairs a white noise auditory conditioning tone (CS), with a noxious foot shock (US), separated by a 30 sec interval (trace) (Figure 2.2A). As can be clearly seen in Figure 2.2B, repetitive presentation of CS-trace-US trials induces robust freezing behavior in response to the CS by the end of conditioning, and in a novel context 48 h later (baseline: 13 ± 7 % time spent freezing; last conditioning CS: 77 ± 10 % time spent freezing; 1st test CS : 59 ± 6.6 % time spent freezing, $F_{2,30} = 16.81, P < 0.001$). To determine if membrane bound AMPA receptor expression is altered in the ACC in response to trace fear conditioning, the ACCs of adult (8-12 wks) C57 mice were extracted immediately after conditioning, and analysed through Western blot analysis (Figure 2.2C). The expression levels of membrane AMPA receptor GluA1 subunits were measured in response to four conditions: trace fear conditioning (10 X CS-trace-US), shock only (10 X US), delay fear conditioning (10 X CS-US), or exposure to the conditioning chamber. A one way ANOVA revealed that trace fear conditioning induced a significant upregulation of membrane bound GluA1 subunits in the ACC compared to all three other forms of conditioning (chamber: 1 ± 0.02, n = 8; trace fear: 1.19 ± 0.05 times the chamber alone value, n = 8; shock: 1.00 ± 0.07 times the chamber alone value, n = 6; delay: 1.05 ± 0.07 times the chamber alone value, n = 8; $F_{3,26} = 6.70, P < 0.05$; Figure 2.2D). Importantly, total GluA1 levels within the ACC remained unchanged in mice exposed to trace fear conditioning (chamber: 1 ± 0.07, n = 6; shock: 0.98 ± 0.08 times the chamber alone value, n = 4, trace fear: 1.02 ± 0.06, n = 6; $F_{2,13} = 0.06, P > 0.05$; Figure 2.2E), and similar levels of membrane bound GluA2/3 were also observed (chamber: 1 ± 0.05, n = 5; shock: 1.0 ± 0.05 times the chamber alone value, n
= 4; trace fear: $0.97 \pm 0.05$, n = 5; $F_{2,11} = 0.11$, $P > 0.05$; Figure 2.2F). These results suggest that trace fear learning may be mediated through rapid upregulation of ACC synaptic AMPA receptors containing the GluA1 subunit.
Figure 2.2: Trace fear upregulates membrane AMPA receptor GluA1 subunits within the ACC. (A) We exposed adult (8-12 wks) C57 mice to one of 4 conditions: trace fear conditioning, delay fear conditioning, shock only, or exposure to the chamber (all treatments lasted 43 min). (B) 48 h later, in a novel environment, mice exposed to trace fear conditioning exhibit robust freezing behavior in response to the CS. (C) We performed western blot analysis of ACC samples extracted immediately following conditioning. (D) Mice exposed to trace fear conditioning show a significant upregulation of membrane bound GluA1 in the ACC (chamber: n = 8; trace fear: n = 8; shock: n = 6; delay: n = 8; $F_{3,26} = 6.70$). (E) Total GluA1 levels within the ACC were not affected by trace fear conditioning (chamber: n = 6; shock: n = 4, trace fear: n = 6; $F_{2,13} = 0.06$). (F) Membrane bound GluA2/3 expression levels were not affected by trace fear conditioning (chamber: n = 5; shock: n = 4, trace fear: n = 5; $F_{2,11} = 0.11$). (* $P < 0.05$)

2.3.3 Activation of NMDA receptor GluN2B subunits is required for the induction of trace fear memory

Postsynaptic AMPA receptor insertion is believed to be a critical component of ACC LTP (Xu et al., 2008). In the hippocampus, NMDA receptor activation can drive AMPA receptor insertion in the postsynaptic membrane of pyramidal neurons (Shi et al., 1999). As LTP is believed to be the cellular mechanism of learning and memory, and is also modulated in vitro in the ACC by GluN2B subunits, the next set of experiments set out to investigate if NMDA receptor GluN2B subunit activity is necessary for trace fear
memory and learning induced AMPA receptor upregulation. Behavioral and western blot analyses were performed on separate groups of adult mice with systemic pharmacological inhibition of either Ro25-6981 or Ifenprodil, two selective GluN2B antagonists that have been repeatedly used to assess GluN2B function (Zhao et al., 2005; Zhang et al., 2008b). In the first set of experiments, intraperitoneal (i.p.) injections of Ro-25-6981 (10 mg/kg), Ifenprodil (10 mg/kg), or saline, were performed 30 min prior to trace fear conditioning, and freezing behavior was assessed in response to the CS in a new context 48 h later (Figure 2.3A). There was no difference in freezing behavior during trace fear conditioning between mice exposed to saline, Ro25-698, or Ifenprodil prior to training ($F_{1,12} = 0.39$; Figure 2.3B). In contrast, tests in a new context 48h later revealed significant differences in freezing behavior in response to presentation of the CS. Specifically, mice treated with either Ro25-6981 or Ifenprodil prior to training exhibited a significant reduction in freezing behavior compared to mice exposed to saline or Ro25-6981 30 min prior to testing (saline: 56 ± 7 %, n = 7; Ro25-6981: 19 ± 9 %, n = 8; ifenprodil: 23 ± 4 %, n = 6; Ro25-6981 at test: 49 ± 10%, n = 5; $F_{3,22} = 6.64$, $P < 0.05$; Figure 2.3C), indicating that GluN2B activity during learning is necessary for trace fear memory formation.

2.3.4 Trace fear learning induced membrane GluA1 upregulation is GluN2B dependent

The next set of experiments investigated if treatment with Ro25-6981 prior to trace fear conditioning affects learning induced upregulation of membrane GluA1 in the ACC. Mice were first exposed to i.p. injections of Ro25-6981 (10 mg/kg i.p.), and then
were either exposed to trace fear conditioning, or to the chamber alone. Remarkably, treatment with Ro25-6981 prior to training almost completely blocked trace fear induced membrane GluA1 upregulation within the ACC (chamber: 1.0 ± 0.1, n = 6; trace fear: 1.1 ± 0.1 times the chamber alone value, n = 6; P > 0.05 Figure 2.3D), indicating that the downstream target of learning induced GluN2B activity is the AMPA receptor GluA1 subunit. Importantly, the effects of Ro25-6981 treatment on trace fear learning were not due to any analgesic effects that may impair CS-US associations, as evidenced by similar nociceptive thresholds of mice exposed to Ro25-6981 or saline in hot plate (saline: 7.29 ± 0.4 s, n = 6; Ro25-6981: 6.45 ± 0.3 s, n = 6, P > 0.05) and tail flick tests (saline: 6.88 ± 0.2 s, n = 6; Ro25-6981: 6.70 ± 0.3 s, n = 6, P > 0.05; Figure 2.3E). Taken together, these results indicate that trace fear memory formation requires conditioning induced, NMDA receptor mediated, upregulation of GluA1 subunits within the ACC.
Figure 2.3: NMDA receptor GluN2B subunit activity is required for trace fear memory. (A) We exposed mice to i.p. injections of either Ro25-6981 or ifenprodil (10 mg/kg @ 2 mg/ml), or saline, 30 min prior to trace fear conditioning and assessed...
freezing behavior in response to the CS in a new context 48 h later. (B) Mice exposed to either Ro25-6981 or ifenprodil prior to training exhibited normal learning behavior but (C) displayed a robust reduction in freezing behavior in response to the CS compared to mice exposed to saline, or to mice exposed to Ro25-6981 30 min prior to testing (saline: n = 7; Ro25-6981: n = 8; ifenprodil: n = 6; Ro25-6981 at test: n = 5; F_{3,22} = 6.64). (D) Treatment with Ro25-6981 30 min prior to training blocked trace fear induced membrane GluA1 upregulation in the ACC. (E) Mice displayed similar response latencies in the hot plate (55˚C) and tail flick assays when treated with Ro25-6981 (n = 6) or saline (n = 6) 30 min before testing. (* P < 0.05)

2.3.5 *GluN2B subunits within the ACC are required for trace fear memory*

To determine if NMDA receptor GluN2B subunit activity within the ACC is necessary for trace fear learning, bi-lateral cannulae were implanted in the ACC of adult mice in order to administer pharmacological antagonists within this specific brain region (Li et al., 2010). After a two week recovery period, mice were exposed to bilateral 0.5 µl infusions of either Ro25-6981 (2 µg/µl) or saline 15 minutes prior to trace fear training. In accordance with the above results, both groups showed similar learning curves (Figure 2.4A), but when tested in a new context 48 h later, mice exposed to Ro25-6981 prior to training showed a significant reduction in freezing behavior in response to the CS compared to mice exposed to saline (saline: 52 ± 6%, n = 8; Ro25-6981: 24 ± 9%, n = 8; t = 2.49, P < 0.05; Figure 2.4B). In addition, to examine if this effect extended to remote fear memory, freezing behavior was again assessed in these mice one month
after trace fear conditioning. Remarkably, the fear memory impairment was still evident when tested one month after training (saline: 26 ± 8%; Ro25-6981: 7 ± 2%; \( t = 5.11, P < 0.05 \); Figure 2.4C). These results show that NMDA receptor GluN2B subunits within the ACC are necessary for trace fear learning, and suggest that long-term memory consolidation processes are disrupted by blocking early NMDA mediated mechanisms within the ACC during learning.

Figure 2.4: GluN2B subunit activity within the ACC is necessary for trace fear learning. (A) Mice exposed to bilateral 0.5 µl infusions into the ACC of either Ro25-
or saline 15 minutes prior to trace fear training exhibited similar learning curves. (B) Mice exposed to Ro25-6981 prior to training showed a significant reduction in freezing behavior in response to the CS when tested in a new context 48 h later (saline: n = 8; Ro25-6981: n = 8, t = 2.49). (C) (Left) The memory impairment was still evident one month after training (saline: n = 4; Ro25-6981: n = 4, t = 5.11). (Right) Representative markers indicating microinjection locations. (* P < 0.05)

2.3.6 CP-AMPAR activity within the ACC is necessary for trace fear memory consolidation

Western blot data revealed that trace fear conditioning rapidly upregulates postsynaptic AMPA receptor GluA1, but not GluA2, subunits within the ACC. Given that CP-AMPAR receptors are GluA2 lacking, and are upregulated by in vivo experience in the mouse barrel cortex (Clem et al., 2008) and the lateral amygdala (Clem and Huganir, 2010), we next sought to determine if CP-AMPAR activity within the ACC immediately following conditioning was necessary for long term memory consolidation. Adult C57 mice with bi-lateral ACC cannulae were exposed to trace fear conditioning, and immediately after training received intra-ACC microinjections of the CP-AMPAR antagonist, 1-naphthylacetyl spermine (NASPM) (3 mM, 0.5 µl/side) or saline (0.5 µl/side; Figure 2.5A-B). Remarkably, when assessed for memory retrieval twenty-four hours later in a new context, mice exposed to intra-ACC NASPM injections immediately after conditioning displayed significantly less freezing behavior in response to the CS than mice exposed to saline (saline: 53 ± 8 %, n = 6; NASPM: 16 ± 5 %, n = 6; P < 0.05;
Figure 2.5C). In combination with the biochemical and behavioral data, these observations strongly indicate that trace fear learning involves rapid CP-AMPAR trafficking within the ACC, and that this process is necessary for subsequent memory consolidation.

**Figure 2.5:** ACC CP-AMPAR receptors are necessary for trace fear memory. (A) Immediately following trace fear conditioning, we exposed mice to bi-lateral, intra-ACC microinjections of either NASPM (3 mM, 0.5 µl/side) or saline (0.5 µl/side). (B) Learning curves. (C) (Left) When tested in a new environment 24 h later, mice exposed to intra-
ACC injections of NASPM displayed significantly less freezing behavior in response to the CS (saline: n = 6; NASPM: n = 6, t = 3.6). (Right) Representative markers indicating microinjection locations. (* P < 0.05)

2.3.7 Trace fear learning rapidly induces functional CP-AMPA receptors within the ACC

In order to determine if the rapid membrane bound GluA1 upregulation observed within the ACC of mice exposed to trace fear conditioning corresponds to functional CP-AMPARs, we assessed the contribution of CP-AMPA channels to excitatory postsynaptic currents (EPSCs) observed in layer II/III pyramidal neurons through whole-cell patch-clamp recordings (Figure 2.6A). We used adult transgenic mice in which the expression of green fluorescent protein (GFP) is controlled by the promoter of the c-fos gene (Barth et al., 2004; Clem et al., 2008; Li et al., 2010). C-fos is an activity dependent gene that can be used as an indicator of recent neuronal activity (Han et al., 2003; Frankland et al., 2004). This method thus allowed us to record from recently activated neurons (FosGFP positive), whilst performing observations from neighboring neurons in the same slice that were not activated (FosGFP negative), allowing for robust within subjects comparisons (Figure 2.6B-C). Previous work with these transgenic mice revealed that GFP expressing neurons within the ACC express changes in excitatory transmission in response to neuropathic pain, and that these changes are not present in GFP negative neurons (Li et al., 2010). We tested the effects of NASPM on EPSCs of FosGFP positive and FosGFP negative pyramidal neurons within the ACC of mice exposed to trace fear conditioning. We observed that
NASPM markedly reduced the amplitude of FosGFP positive neuronal EPSCs to 67% of the baseline (Figure 2.6D-E), an attenuation that was significantly greater than that observed on FosGFP negative neurons (FosGFP positive: 67.2 ± 5.9 %, n = 6; FosGFP negative: 93.95 ± 5.13 %, n = 9; P < 0.05, Figure 2.6F). The increased NASPM sensitivity observed in ACC pyramidal neurons from trace fear conditioned mice indicates an increase in active CP-AMPA receptors (Clem and Huganir, 2010), and indicates that trace fear conditioning induces rapid CP-AMPAR trafficking within the ACC.
Figure 2.6: Trace fear memory is mediated by postsynaptic CP-AMPAR trafficking within the ACC. (A) (Left) We performed whole cell patch clamp recordings immediately following trace fear conditioning in ACC slices from transgenic FosGFP mice. (Right) We recorded EPSCs from pyramidal neurons in layer II/III whilst stimulating layers V/VI of the ACC. (B) Trace fear conditioning induces c-Fos activity
within the ACC; scale bar represents 100 µm. (C) Representative images of whole-cell patch clamp recordings of FosGFP negative pyramidal neurons (top panels), and FosGFP positive pyramidal neurons (bottom panels) as indicated by yellow showing overlap between GFP and dye loaded pipette; scale bar represents 20 µm. (D) Representative traces of single pyramidal neuron recordings before “a” and after “b” NASPM application. (E-F) NASPM significantly inhibited the amplitude of FosGFP positive neuronal EPSCs to 67% of the baseline, a reduction that is significantly greater than that observed on FosGFP negative neurons (FosGFP positive: n = 6; FosGFP negative: n = 9, t = -2.38). ** Data for this figure was collected by Drs. Xiang-yao Li and Tao Chen.

2.4 Discussion

The present study is the first to demonstrate that rapid LTP-like events occur within the ACC during trace fear learning. Several observations support this finding: First, trace fear conditioning induced an upregulation of membrane bound GluA1 within the ACC that is evident immediately after conditioning. Second, NMDA receptor GluN2B subunit activity within the ACC was found to be critical for trace fear learning, and disruption of these receptors during conditioning prevented AMPA receptor GluA1 subunit upregulation and fear memory formation. Furthermore, blockade of CP-AMPAR activity immediately following trace fear conditioning was sufficient to prevent trace fear memory retrieval 24h later. Accordingly, through the use of transgenic FosGFP mice, trace fear learning was found to recruit CP-AMPARs in c-Fos expressing ACC.
pyramidal cells. These findings show that early memory formation occurs within the
cortex during trace fear learning, and identifies a critical, rapid synaptic strengthening
mechanism that is necessary for consolidation of long term fear memory.

2.4.1 Trace fear learning induces immediate membrane GluA1 upregulation

A key component of LTP induction is the upregulation of postsynaptic AMPA
receptors (Song and Huganir, 2002), and AMPA receptor plasticity is strongly implicated
in learning and memory (Rumpel et al., 2005; Kessels and Malinow, 2009). The data
presented here shows a rapid increase in membrane GluA1 subunit protein in the ACC
of mice extracted immediately after in vivo trace fear learning. This is consistent with
recent observations that trace eye blink conditioning induces changes in neuronal firing
within the mPFC (Takehara-Nishiuchi and McNaughton, 2008). These findings support
the notion that experience dependent synaptic activity can ‘tag’ specific synapses for
subsequent changes in excitatory transmission (Frey and Morris, 1997), and may
represent similar mechanisms observed in the hippocampus (Matsuo et al., 2008).

Previous investigations of fear learning have identified changes in excitatory
transmission within the hippocampus and amygdala (McKernan and Shinnick-
Gallagher, 1997; Rogan et al., 1997; Rumpel et al., 2005; Matsuo et al., 2008; Migues
et al., 2010), and increases of GluA1 in dendritic spines of CA1 neurons have been
observed 24 h after contextual fear conditioning (Matsuo et al., 2008). These results
therefore suggest that fear learning is mediated through a complex interplay between
various brain areas, and that rapid plasticity within the cortex is in itself a mediator of
learning induced alterations that are required for long term memory consolidation. Indeed, the recent findings that CaMKIV is required for translation-dependent early synaptic potentiation within the ACC (Toyoda et al., 2010), and that trace fear memory is enhanced in mice overexpressing CaMKIV (Wu et al., 2008), suggest that targeting these early cortical changes induced by learning can alter the strength of the consolidation of the fear memory.

2.4.2 NMDA dependent AMPA GluA1 upregulation

If LTP mechanisms in the ACC mediate trace fear learning, then modulators of ACC LTP observed in vitro should affect trace fear learning. Indeed, the pharmacological experiments presented show that in vivo blockade of NMDA receptor GluN2B subunits during trace fear conditioning prevented fear memory recall, and blocked the upregulation of membrane bound GluA1; indicating that GluA1 subunits are the downstream target of experience dependent GluN2B activity. This is in accordance with previous observations that genetic GluN2B overexpression can enhance fear memory acquisition (Tang et al., 1999). Although LTP has long been considered to be the neural substrate for learning and memory (Kandel, 2001a), and reports have shown that NMDA GluN2B subunit activity is critical for ACC LTP (Toyoda et al., 2005), and that AMPA receptor insertion corresponds to potentiation of excitatory synaptic transmission (Gu et al., 1996; Boehm et al., 2006; Clem and Barth, 2006), this is the first evidence that in vivo trace fear learning induces rapid GluN2B mediated AMPA receptor insertion within the cortex. Indeed, although various publications implicate
NMDA receptors in several brain regions in fear memory, including the amygdala (Zhang et al., 2008b), hippocampus (Huerta et al., 2000; Zhang et al., 2008a), and forebrain (Tang et al., 1999), studies had yet to identify the learning related downstream target. In addition, studies have questioned the requirement of NMDA GluN2B receptors in hippocampal LTP and learning (Huang and Malenka, 1993; Liu et al., 2004). These findings highlight that there is a critical cortical contribution to fear learning, and that early GluN2B dependent plasticity within the cortex is necessary for long term memory formation.

2.4.3 CP-AMPAR activity within the ACC is necessary for memory consolidation

Mounting evidence indicates that AMPA receptor trafficking is a critical component of synaptic strengthening, and may underlie learning (Shi et al., 1999; Song and Huganir, 2002; Kessels and Malinow, 2009). Accordingly, the results here show that in vivo blockade of CP-AMPARs in the ACC immediately following trace fear conditioning robustly blocked memory retrieval 24h later. In combination with the biochemical and behavioral data, these findings indicate that rapid CP-AMPAR receptor upregulation during conditioning is necessary for long term memory consolidation, and is the first account that early memory formation within the ACC is necessary for long term retrieval. Several studies support the recruitment of CP-AMPARs in synaptic strengthening (Clem and Barth, 2006; Plant et al., 2006; Guire et al., 2008), and CP – AMPAR trafficking was found to be induced by cocaine exposure in vivo (Bellone and Luscher, 2006). In particular, in the CA1 region of the hippocampus, different LTP
induction protocols have been demonstrated to induce transient increases of CP-AMPARs, including theta burst stimulation (Guire et al., 2008), and pairing protocol (Plant et al., 2006). Of particular interest are previous in vitro observations in the CA1 region of the hippocampus, where LTP induced by a pairing protocol corresponded to rapid CP-AMPAR upregulation (Plant et al., 2006) that lasted less than 25 minutes, and was NMDA receptor dependent. Importantly, LTP induction corresponded to increases in sensitivity to the CP-AMPAR channel blocker, polyamine toxin philanthotoxin 433 (PhTx). Remarkably, there was a lack of LTP recovery if PhTx was applied immediately after LTP induction, suggesting that activation of new CP-AMPARs immediately after LTP induction is necessary for subsequent potentiation of excitatory transmission, further indicating that GluA1 trafficking is necessary for subsequent plasticity related events. Accordingly, in the present study, whole cell patch clamp recordings of FosGFP neurons indicated that trace fear conditioning potentiates postsynaptic CP-AMPAR activity in recently activated layer II/III ACC neurons. These ex vivo observations correspond well with previous in vitro pharmacological experiments in the ACC where LTP was reduced by PhTx blockade of CP-AMPARs 5 min after paired training, despite an absence of PhTx effect on basal responses (Toyoda et al., 2007a). The present findings thus indicate that trace fear learning induces LTP-like CP-AMPAR upregulation in the ACC. Several studies indicate that storage for a given memory is mediated by a select population of neurons (Rumpel et al., 2005; Clem and Barth, 2006; Han et al., 2009). A possible interpretation of the data is that upregulated CP-AMPARs may function as a ‘tag’ for subsequent memory consolidation processes, supporting the hypothesis that activity-dependent synaptic ‘tagging’ may mediate stabilization of LTP
(Frey and Morris, 1997), and suggests that such mechanisms are rapidly engaged within the cortex during trace fear learning.

Recordings were focused on layer II/III neurons as there is strong evidence that thalamic-ACC evoked potentials extend through layer V/VI of the ACC and into layer II/III (Lee et al., 2007b). Furthermore, ACC pyramidal neurons in layer II/III undergo changes in excitatory transmission in response to LTP induction protocols and chronic pain (Toyoda et al., 2007a; Xu et al., 2008; Li et al., 2010). More importantly, through recordings of layer II/III pyramidal ACC neurons, enhanced LTP was found in mice overexpressing CaMKIV, which corresponded with enhancements in trace fear learning (Wu et al., 2008).

2.5 Conclusions

In summary, these findings indicate that early reorganisation within the ACC is critical for trace fear memory consolidation. The findings suggest that trace fear learning is mediated through rapid excitatory potentiation within the ACC, and supports the notion that experience dependent synaptic activity can ‘tag’ specific synapses for subsequent changes in excitatory transmission (Frey and Morris, 1997). Furthermore, the present results show strong evidence that such rapid potentiation is necessary for consolidation, suggesting that experience induced CP-AMPAR activity mediates memory stabilization within the cortex. Consolidation theory suggests that learning induces an initial rapid and transient strengthening of the connections between the medial temporal lobe and cortical areas, whilst alterations of cortico-cortical connections
are slower but long-lasting (Frankland and Bontempi, 2005). Previous studies, as well as retrograde labelling data presented above, show evidence of amygdala-ACC projections. The present data thus suggests that rapid strengthening of amygdala-cortical connections during trace fear learning can occur, and requires rapid LTP-like changes (Figure 2.7).

**Figure 2.7:** Proposed model for trace fear learning induced AMPAR upregulation. During trace fear conditioning, Ca\(^{2+}\) influx via NMDARs initiates AMPAR upregulation through activation of Ca\(^{2+}\)/calmodulin-dependent protein kinase IV (CaMKIV) related pathways. Newly recruited postsynaptic AMPA receptors help potentiate glutamatergic
excitatory transmission within the ACC, establishing plasticity in neuronal populations for long term memory storage.
CHAPTER 3

GENETIC ENHANCEMENT OF NEUROPATHIC AND INFLAMMATORY PAIN BY FOREBRAIN UPREGULATION OF CREB-MEDIATED TRANSCRIPTION
CHAPTER 3: GENETIC ENHANCEMENT OF NEUROPATHIC AND INFLAMMATORY PAIN BY FOREBRAIN UPREGULATION OF CREB-MEDIATED TRANSCRIPTION

3.1 Background

Pain is a sensation that alerts us to physical harm and is associated with unpleasant emotions. The ACC plays a major role in the emotional (affective) component of pain (reviewed in chapter 1). This is supported by human neuroimaging data showing that ACC activity is correlated with the unpleasantness of pain (Rainville et al., 1997), and patients with lesions in the ACC that show altered emotional responses to pain (Rainville et al., 1997; Zhuo, 2008)(Rainville et al., 1997; Zhuo, 2008)(Rainville et al., 1997; Zhuo, 2008)(Rainville et al., 1997; Zhuo, 2008)(Bushnell et al., 2013). Furthermore, animal studies show the some ACC neurons can be activated by noxious stimulation on any part of the body surface, as opposed to being topographically organized like the S1 and S2 cortical areas, further exhibiting evidence of a role for the ACC that expands beyond attention to the area involved. This chapter will highlight the current literature exploring cortical mechanisms involved in chronic pain development. Long term potentiation of ACC synaptic transmission is believed to be a key mechanism for chronic pain development and maintenance. In vitro studies have shown that such long term enhancements in ACC excitatory transmission require protein synthesis, and are sensitive to transcription and
translation inhibitors. This chapter will assess the role of forebrain, CREB-mediated transcription in chronic pain behaviors.

3.1.1 Chronic pain behavior

Acute pain experienced under normal physiological conditions is typically characterized by high thresholds and is transient (Basbaum et al., 2009). In contrast, chronic pain resulting from peripheral tissue damage and inflammation (inflammatory pain), or from lesions to the nervous system (neuropathic pain) is characterized by persistent nociceptive hypersensitivity (Ji et al., 2003; Zhuo, 2008). In the laboratory setting, two types of injuries are employed to investigate the mechanisms underlying chronic pain. Inflammatory pain models use subcutaneous injections into the hindpaw of inflammatory agents such as formalin or complete Freund’s adjuvant (CFA), resulting in tissue injury, whereas neuropathic pain models are commonly caused through surgical injury of a peripheral nerve (Woolf and Salter, 2000). These have proven to be robust models for the study of chronic pain and have helped researchers yield much insight into the molecular mechanisms of chronic pain. Chronic inflammatory and neuropathic pain can manifest in changes in behavioral responses to tactile and thermal stimuli that are easily detectable in animal models. Human observers can reliably detect and measure two major forms of sensitized behavioral responses: hyperalgesia, the enhancement of nociceptive responses to noxious stimuli, and allodynia, behavioral nociceptive responses to previously innocuous stimuli (Chaplan et al., 1994; Vadakkan et al., 2005) (Figure 3.1).
Figure 3.1: Behavioral sensitization to nociceptive stimuli. Chronic pain manifests in two major forms of sensitized behavioral responses: hyperalgesia, the enhancement of nociceptive responses to noxious stimuli, and allodynia, behavioral nociceptive responses to previously innocuous stimuli.

Mechanical allodynia is reliably assessed in animal models and easily detected as a decrease in nociceptive threshold that accompanies chronic pain, such that stimuli that would otherwise be innocuous will evoke noxious nociceptive behavior (Toyoda et al., 2009a). Mechanical thresholds can be assessed by observing the responsiveness of hindpaws to the application of von Frey filaments, which allow investigators to apply accurate and constant force to specific areas of the skin. Previous reports have identified that the normally innocuous 0.4 mN (No. 2.44) filament yields consistent nociceptive responses in mice exposed to chronic pain models (Wei et al., 2002b).
These phenomena reflect similar sensitization of pain seen in human patients (Woolf and Mannion, 1999), and may correspond to changes of synaptic transmission in the spinal cord dorsal horn (Sandkuhler and Liu, 1998) or pain related cortical areas (Zhuo, 2008).

3.1.2 Chronic pain behavior corresponds with long term enhancements in excitatory transmission observed in vitro

Through the use of animal models, studies have begun to identify molecular and cellular alterations critical for chronic pain. In the spinal cord, neuropathic injuries can induce long-term abnormal neural activity along primary afferent pathways (Basbaum et al., 2009). For example, in vitro recordings of dorsal horn neurons from animals with chronic neuropathic pain have shown potentiated excitatory responses and decreases in firing thresholds in (Sandkuhler and Liu, 1998; Ikeda et al., 2006a; Sandkuhler, 2007). Similarly, repetitive squeezing of the sciatic nerve induces LTP at C-fibre synapses (Sandkuhler, 2007), and previously sub-threshold synaptic input can induce action potentials in dorsal horn neurons (Latremoliere and Woolf, 2009). Unfortunately, chronic pain persists beyond the duration of injury, and several lines of evidence indicate that long term changes in cortical mechanisms may be responsible for chronic pain development. Indeed, even lesions of spinal pathways cannot always provide relief to chronic pain patients. Moreover, the focus of this thesis is on cortical molecular mechanism, and as such this section will focus on cortical mechanisms.
More recently, changes in cortical neural processing corresponding to chronic pain conditions have begun to be investigated. Neuroimaging data of chronic pain patients shows that the ACC is activated by peripheral application of previously innocuous stimuli (Bushnell, 2013). Correspondingly, animal studies have also shown robust behavioral nociceptive sensitization in chronic pain models that correspond with enhancements of excitatory transmission in ACC synapses. For example, Western blot analysis of ACC samples extracted from mice with chronic neuropathic pain show upregulation of phosphorylated AMPA receptor GluA1 subunits, and electrophysiological recordings showed enhanced GluA1 mediated postsynaptic responses in layer II/III neurons within the ACC (Xu et al., 2008). Thus, similar to in vitro observations of ACC LTP, behavioral nociceptive sensitization corresponds to increases in AMPA receptor GluA1 subunits, perhaps reflecting potentiated excitatory transmission in nociceptive areas. Similarly, NMDA receptor subunits identified as critical modulators of ACC LTP appear to also play important roles in chronic pain. For example, transgenic mice with forebrain-targeted GluN2B over-expression display enhanced responses to inflammatory pain, whereas behavioral responses to acute pain were unaffected (Wei et al., 2001). Accordingly, Western blot results show that peripheral tissue inflammation induced by CFA results in an upregulation of GluN2B subunit receptors within the ACC (Wu et al., 2005b), that corresponded with increases in GluN2B mediated EPSCs in layer II/III pyramidal neurons, as shown by in vitro whole cell patch clamp recordings from ACC slices. Additionally, they also demonstrated that local ACC micro-injection of the GluN2B selective antagonist, Ro25-6981 attenuated
behavioral allodynia. Remarkably, enhanced postsynaptic excitatory potentials were also observed *in vivo* in mice with CFA compared to saline injections.

3.1.3 Injury triggered gene expression

Chronic pain corresponds with persistent potentiation of excitatory transmission at ACC synapses (Zhuo, 2008), and activity mediated gene expression has been suggested to be a critical link between NMDA mediated intracellular activity and AMPA receptor upregulation, the consequence of which is LTP. Various reports indicate that neuropathic injury triggers intracellular signaling cascades that can induce persistent molecular changes which lead to potentiation of glutamatergic excitatory transmission (Wu et al., 2005c; Zhao et al., 2006; Xu et al., 2008). Amputation or even strong peripheral stimulation of the hindpaw in rats induces activation of plasticity-related immediate early genes, including Egr1, CREB, and c-Fos within ACC neurons (Wei et al., 1999; Wei et al., 2002b). Activity-dependent gene expression is important for long term changes in synaptic transmission (Vignes and Collingridge, 1997; Kandel, 2001b). Increases in concentration of intracellular Ca$^{2+}$ activates various intracellular signaling cascades, including cAMP and CaMKIV dependent pathways (Silva et al., 1998). In turn, these pathways lead to the phosphorylation of the transcription factor CREB at the serine 133 site (Sheng et al., 1991)(Reviewed in Chapter 1). CREB is a major transcription factor believed to play a critical role in the formation of long-term memory (Bourtchuladze et al., 1994; Silva et al., 1998; Kida et al., 2002). Consistent with this hypothesis, genetic enhancement of forebrain CREB corresponds with improvements in
long term memory and enhanced LTP in the CA1 region of the hippocampus (Suzuki et al., 2011).

Although previous studies have shown evidence of injury-induced CREB activation (phosphorylation) in cortical areas (Wei et al., 2002b), no report has shown direct evidence that CREB-mediated transcription in the cortex actually contributes to behavioral sensitization. If chronic pain corresponds with injury induced, transcription mediated LTP, enhancements of transcription should also enhance chronic pain. To address this question, chronic pain development was measured in transgenic mice expressing dominant active CREB mutant in the forebrain (Y134F) driven by the αCaMKII promoter (Suzuki et al., 2011), which is expressed predominantly in forebrain regions, including cortical areas, the amygdala, and hippocampus (Mayford et al., 1996). Previous reports indicate that these mice show increased CREB activity to sensory stimuli, and exhibit enhancements in long term memory and LTP in the CA1 region of the hippocampus (Suzuki et al., 2011). This chapter will present evidence that enhanced forebrain CREB activity leads to the potentiation of behavioral responses to non-noxious stimuli after injury.

3.2 Methods

3.2.1 Animals
Adult (8 - 12 month) transgenic mice Y134F line C, expressing dominant active CREB in the forebrain, and age matched WT littermates were used for all studies as reported previously (Suzuki et al., 2011). Genotypes were identified by PCR analysis of genomic DNA extracted from mouse ear tissue. All mice were housed under a 12 h light/dark cycle with food and water provided *ad libitum*. All mouse protocols are in accordance with National Institutes of Health guidelines and approved by the Animal Care and Use Committee of University of Toronto.

3.2.2 Behavioral experiments

Acute pain assessment was performed as published previously (Wei et al., 2001). We determined the latency of behavioral responses to placement on a thermal hot plate (55°C) (Columbus Instruments, Columbus, OH), and the latency for the spinal nociceptive tail-flick reflex, evoked by radiant heat applied to the underside of the tail (Columbus Instruments, Columbus, OH). All tests were performed blind to genotype. Fifty percent mechanical threshold was assessed with a set of von Frey filaments (Stoelting, Wood Dale, Illinois) using the up-and-down method (Chaplan et al., 1994). Mice were placed in plexiglass containers with elevated wire mesh flooring, and were allowed to acclimate for 30 min before testing. A threshold stimulus was determined by observing animal hind paw withdrawal upon application of a von Frey filament; positive responses included prolonged hind paw withdrawal, or licking or biting of the hind paw. Mechanical allodynia was measured as described previously (Wei et al., 2001), and was assessed with the 0.4 mN (No. 2.44) von Frey filament, previously observed to produce...
minimal hind paw withdrawal in untreated mice (Wei et al., 2001). Positive responses included licking, biting, and prolonged withdrawal of the hindpaw. Experiments consisted of 10 trials, with 10 min inter-trial intervals. All observations were performed blind.

3.2.3 Inflammatory pain models

Formalin (5%, 10 μl; Sigma-Aldrich) or complete Freund’s adjuvant (CFA, 50%, 10 μl; Sigma-Aldrich) was injected subcutaneously into the dorsal side of the left hind paw as reported previously (Wei et al., 2001). In the formalin test, the total time spent licking or biting the injected hind paw was recorded for each five-minute intervals for two hours post injection. In the CFA model, mechanical sensitivity was assessed with the 0.4 mN (No. 2.44) von Frey filament as described above.

3.2.4 Neuropathic pain model

The neuropathic pain model consisted of ligation of the common peroneal nerve (CPN) and was performed as previously described (Vadakkan et al., 2005; Xu et al., 2008). Mice were anesthetized by inhaled isofluorane (1-3%). In the rear leg, a clear depression is visible between the anterior and posterior muscle groups. A 1 cm skin incision was made along this depression from the fibular head directed vertically downward. The CPN is exposed under the posterior group of muscles running almost
transversely. The muscles were pulled laterally and posteriorly to expose the nerve, and the CPN was ligated with sterile chromic gut suture 5-0 (Ethicon, Somerville, NJ) without disturbing or including the blood vessel. The ligature was tightened until contraction of the dorsiflexors on the foot was visible as twitching of the digits. Thereafter, the skin was sutured with sterile 5-0 silk suture.

3.2.5 Data analysis

Results are expressed as mean ± SEM. Unpaired student t-tests and two-way repeated Analyses of Variance (ANOVA) were performed. Significant effects were further tested by the Holm-Sidak test for multiple comparisons. In all cases $P < 0.05$ was considered statistically significant. Sample sizes were determined based on previous reports observing significant changes in nociceptive behaviors in chronic inflammatory and neuropathic pain models (Wang et al., 2011, Li et al., 2010, Wei et al., 2002).

3.3 Results

3.3.1 Acute nociception

In order to determine if forebrain overexpression could affect acute nociception, wild-type (WT) and transgenic (Y134F) mice were first exposed to the hot plate (55 °C) and tail flick tests of thermal nociception. Both groups of mice showed similar response
latencies to the hot plate (WT: 7 ± 1 sec, n = 7; Y134F: 8 ± 1 sec, n = 7, P > 0.05; Figure 3.1A) and tail flick tests (WT: 6 ± 1 sec, n = 7; Y134F: 6 ± 1 sec, n = 7, P > 0.05; Figure 3.1B). Acute pain is physiological, with an obvious advantageous survival purpose. This form of pain is insensitive to genetic or pharmacological inhibition of Ca\textsuperscript{2+}-calmodulin dependent intracellular pathways (Wei et al., 2002b; Wang et al., 2011). Accordingly, both groups of mice also showed similar 50% mechanical response thresholds (WT: 0.7 ± 0.1 g, n = 7; Y134F: 0.7 ± 0.1 g, n = 7, P > 0.05; Figure 3.1C). These results indicate that CREB overexpression in the forebrain does not alter basal nociceptive thresholds.

**Figure 3.1: Behavioral assessment of acute pain.** A) Acute nociception was unaltered by forebrain CREB overexpression. Response latencies in the hot plate and B) tail flick tests were similar between groups. C) 50% mechanical threshold levels were indistinguishable between groups.
3.3.2 Acute inflammatory pain

The effects of forebrain CREB overexpression on acute inflammatory pain were assessed by measuring behavioral (licking) responses to inflammation brought on by intradermal injections of formalin (5%) to the hindpaw (Wei et al., 2001). Transgenic mice with forebrain NMDA receptor subtype GluN2B overexpression show a robust enhancement of acute inflammatory pain (Wei et al., 2001); whereas transgenic mice with a genetic deletion of adenylyl cyclase type 1 (AC1) show reduced behavioral nociceptive responses to peripheral injection of formalin (Li et al., 2010; Wang et al., 2011). Within the first 10 min after formalin injection, behavioral responses were undistinguishable between WT and Y134F mice (Figure 3.2A). In the subsequent phases of the test, however, Y134F mice showed a marked reduction in responses, which lasted well into 2 hrs post injection. Indeed, a repeated measures two-way ANOVA revealed a significant main difference in licking time between WT and Y134F mice, and showed a significant interaction between group and time, whereby Y134F mice showed significantly less licking behavior only during phases 2 and 3, but not 1 (Phase 1: WT: 59 ± 20 sec, n = 6; Y134F 45 ± 10 sec, n = 7; Phase 2: WT: 454 ± 71 sec, n = 6; Y134F 288 ± 34 sec, n = 7; Phase 3: WT: 339 ± 68 sec, n = 6; Y134F 141 ± 34 sec, n = 7; group X time interaction, $F_{2,22} = 7.6$, $P < 0.001$; Figure 3.2). The lack of difference during phase one is consistent with the observation that acute behavioral responses to noxious stimuli were not affected in the same mice. In the later stages of the test however (Phase 2 and 3), we saw a marked reduction in licking behavior. These results however differ from previous reports in mice with NR2B forebrain
overexpression, indicating that genetic manipulation at downstream signaling targets may cause different phenotypes (Wei et al., 2001). Future studies are needed to investigate the exact mechanism. This finding also raises the possibility that cortical CREB activity may not directly contribute to behavioral responses in cases of acute inflammation.

Figure 3.2: Behavioral assessment of inflammatory pain. A) Formalin test, Y134F mice (n = 6) show significantly less licking responses than WT mice (n = 7). B) A significant interaction was detected, whereby Y134F mice only showed decreases in Phase 2 (10 – 60 min) and 3 (60 – 120 min), but not Phase 1 (0 – 10 min) of the formalin test, $F_{2,22} = 7.6$. Phase 1 corresponds with acute inflammatory pain, whereas Phase 2 and 3 represent more tonic states of pain. * $P < 0.05$
3.3.3 Neuropathic pain

Nerve injury (neuropathic pain model) activates Ca\(^{2+}\) calmodulin dependent pathways within the ACC (Wei et al., 2002b; Li et al., 2010), a forebrain structure involved in the affective component of pain (Rainville et al., 1997; Zhuo, 2008) (Reviewed in Chapter 1). WT and CREB-Y134F mice were exposed to ligation of the common peroneal nerve, which induces robust chronic pain in mice lasting weeks (Vadakkan et al., 2005), and behavioral responses were tested at one and two weeks post injury. Under normal (baseline) conditions, application of a 0.4mN von Frey filament to the hindpaw does not elicit much of a response from mice; whereas under chronic pain conditions, similar stimulation results in robust and reliable nociceptive responses (Wei et al., 2001). Although no differences could be detected at baseline, a repeated measures two-way ANOVA showed a significant effect of CREB overexpression on neuropathic pain induced mechanical allodynia \((F_{1,12} = 46.44, P < 0.001; \text{Figure 3.3A})\). Y134F mice showed a significant enhancement in mechanical allodynia at 1 and 2 weeks after nerve ligation surgery (1 week: WT: 54 ± 7 %, n = 7; Y134F 82 ± 8 %, n = 7; 2 weeks: WT: 42 ± 7 %, n = 7; Y134F 75 ± 11 %, n = 7). Y134F mice also showed enhanced allodynia in the contralateral (non-injured) hindpaw, which remained significantly higher after 2 weeks (1 week: WT: 34 ± 5 %, n = 7; Y134F 54 ± 11 %, n = 7) (2 weeks: WT: 10 ± 7 %, n = 7; Y134F 41 ± 10 %, n = 7, \(F_{1,12} = 10.25, P < 0.05; \text{Figure 3.3B})\).
3.3.4 Chronic inflammatory pain

Robust mechanical sensitization is induced by hindpaw injections of complete
Freund’s adjuvant (CFA), and it is often employed as a model of chronic inflammatory
pain in the laboratory. To assess the effects of forebrain CREB overexpression on
chronic inflammatory pain behaviors, mechanical allodynia was measured in adult
Y134F and WT mice exposed to injections of CFA (10 µl) in the left hindpaw. Similar to
neuropathic pain, a two way repeated measures ANOVA showed a significant effect of
CREB overexpression on mechanical allodynia after CFA injection ($F_{1,12} = 19.81$, $P <$
0.05; Figure 3.3C). Y134F mice showed robust enhancements of mechanical allodynia
at 24 h (WT: 56 ± 8 %, n = 4; Y134F 88 ± 5 %, n = 4), 3 days (WT: 25 ± 8 %, n = 4;
Y134F 54 ± 13 %, n = 4), and 1 week after CFA injection (WT: 18 ± 7 %, n = 4; Y134F
46 ± 9 %, n = 4) compared to baseline. In the contralateral paw, significant differences
were only detected at 3 days post injection (WT: 13 ± 4 %, n = 4; Y134F 38 ± 11 %, n =
4, $P < 0.05$; Figure 3.3D), although an enhancement trend is clearly visible across all
time points.
Figure 3.3: Behavioral assessment of chronic inflammatory and neuropathic pain.

A) In the neuropathic pain model, Y134F mice (n = 7) showed a significant enhancement in mechanical allostynia at 1 and 2 Weeks post-surgery compared to WT mice (n = 7) ($F_{1,12} = 46.44$). B) Enhanced allodynia was also seen in the uninjected paw but only after 2 weeks. C) In the CFA model of chronic inflammatory pain, Y134F mice (n = 4) showed a significant enhancement mechanical allostynia compared to WT mice (n = 4) that was evident up to 1 Week post CFA application ($F_{1,12} = 19.81$). D) Enhanced allodynia in the uninjected paw was only evident 3 days post injection. * $P < 0.05$
3.4 Discussion

3.4.1 Acute nociception

In conclusion, this data shows that enhancement of CREB activity within forebrain neurons is sufficient to potentiate behavioral responses to sensory stimuli in animal models of chronic inflammatory and neuropathic pain. This is supported by previous reports that synaptic potentiation (LTP) is enhanced in the regions of the hippocampus (Suzuki et al., 2011) and the ACC (Chen et al., unpublished data). Furthermore, observations that basal excitatory synaptic transmission is not affected in the same transgenic mice supports the current findings that acute responses to physiological noxious thermal and mechanical stimuli were unaffected. Accordingly, forebrain CREB overexpression does not affect the first phase of the formalin test, during which behavioral responses represent direct activation of nociceptive pathways. For example, previous observations in the spinal cord have shown formalin-induced CREB phosphorylation in ipsilateral and contralateral dorsal root ganglion (DRG) neurons that reached peak expression at 10 min (Ji and Rupp, 1997), whereas behavioral responses to formalin injections last beyond 1 hr. In contrast, marked reduction of responses were observed in the later phases (2 and 3), which are thought to be mediated by mechanisms involved in central sensitization of nociceptive
transmission (Ji and Rupp, 1997). These reductions were surprising, and caution should therefore be exercised before using CREB as a marker for acute inflammatory pain.

### 3.4.2 Chronic pain

Remarkably, forebrain overexpression of CREB was sufficient to enhance mechanical allodynia in animal models of chronic inflammatory and neuropathic pain. Chronic pain produced through peripheral inflammation or nerve injury corresponds with potentiation of excitatory transmission in ACC synapses (Zhao et al., 2006; Xu et al., 2008; Toyoda et al., 2009a), which is partly induced through increases in postsynaptic AMPA receptor GluA1 subunits (Xu et al., 2008). Gene expression is important for long term changes in synaptic transmission (Vignes and Collingridge, 1997; Kandel, 2001b), and CREB has been implicated in various events that are known to correspond with changes in postsynaptic receptors, including fear learning (Josselyn et al., 2001; Han et al., 2009; Suzuki et al., 2011) and drug addiction (McClung and Nestler, 2007). Furthermore, chronic pain requires the activation of cAMP and Ca\(^{2+}\)-CaM dependent protein kinase pathways, and the disruption of these pathways reduces chronic pain (Wei et al., 2002b; Vadakkan et al., 2006). As chronic pain (Xu et al., 2008) and fear learning (Chapter 2) (Descalzi et al., 2012) have been shown to correspond with increases in postsynaptic GluA1 subunits in the ACC, it is likely that CREB is involved in the pain induced, Ca\(^{2+}\) CaM dependent, upregulation of postsynaptic AMPA GluA1 receptors, and thus contributes to enhancements in excitatory synaptic transmission within the ACC. The ACC receives robust projections from the thalamus and in turn
also projects to thalamic and spinal pathways (Vogt et al., 2005; Chang et al., 2012). Forebrain overexpression of CREB can thus facilitate increases in excitatory transmission within the ACC, and enhance top-down descending facilitation of spinal sensory transmission (Calejesan et al., 2000; Porreca et al., 2002; Zhuo et al., 2002). It is important to note however, that as the mice used in this study express the dominant active CREB mutant transgene in a number of forebrain structures, other forebrain areas may also be involved, such as the insular cortex, amygdala, and hippocampus. Future studies are needed to investigate experience-induced synaptic and structural changes in the transgenic mice.
CHAPTER 4

CORTICAL GLUK1 KAINATE RECEPTORS MODULATE
SCRATCHING IN ADULT MICE
CHAPTER 4: CORTICAL GLUK1 KAINATE RECEPTORS

MODULATE SCRATCHING IN ADULT MICE

4.1 Background

Itch, like pain, is an uncomfortable sensation that strongly elicits a response towards the area involved. Recent investigations into the mechanisms mediating itch transmission have mainly 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 ACC. This chapter presents evidence of cortical modulation of pruritogen-induced scratching behavior.

4.1.1 Itch pathways

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). Histamine and non-histamine dependent itch producing agents (pruritogens) activate receptors on polymodal primary afferents projecting to lamina I (Johanek et al., 2008; Davidson and Giesler, 2010), in turn innervating STT neurons (Davidson et al., 2009). The histaminergic pathway can be activated by tissue damage, allergic reactions, and infection (Ikoma et al., 2006; Davidson and Giesler, 2010). In response to inflammation, mast cells release histamine in peripheral tissue, where it can bind to
histamine receptors located on ascending C-fibres (Jutel et al., 2009; Jeffry et al., 2011). However, antihistamines are not always effective treatments against itch, indicating that histamine independent itch transmission pathways may exist (Papoiu et al., 2012). Accordingly, a robust, antihistamine insensitive itch sensation is induced in humans and monkeys by skin application of spicules of cowhage (Mucuna pruriens), a tropical legume whose active component is the cysteine protease called mucunain, which activates PAR2 receptors (Shelley WB. and RP., 1955). PAR2 receptors are also located on unmyelinated C-fibres and when activated induce a strong itch response (Steinhoff et al., 2003; Johanek et al., 2007; Namer et al., 2008; Reddy et al., 2008). Recent in vivo recordings from non-human primates show that histamine and cowhage activate separate populations of spinothalamic tract neurons (Davidson et al., 2007) that terminate on various thalamic nuclei (Davidson et al., 2012).

4.1.2 Common mechanisms between pain and itch

Pain and itch are warning mechanisms alerting the system to impending physical harm (Ross, 2011). Although it remains debated whether itch and pain are mediated by distinct pathways (Davidson and Giesler, 2010), strong evidence supports they share common mechanisms. For example, despite human (Schmelz et al., 1997) and cat (Andrew and Craig, 2001) observations that some mechanically insensitive, unmyelinated C-fibers were preferentially activated by histamine in the skin, subsequent investigations identified how they can also respond to chemical nociceptive compounds such as bradykinin and capsaicin (Steinhoff et al., 2003). Furthermore, work in monkeys
has shown that spinothalamic tract neurons that can be either activated by the pruritogens histamine or cowhage are also activated by capsaicin and heat (Davidson et al., 2007). More recently, in vivo recordings showed that some thalamic projecting neurons can be activated by both pain and itch (Davidson et al., 2012), and in particular although some STT neurons were activated by either cowhage or histamine, all pruritogen stimulated neuron were mechanically activated.

Despite our strong understanding of integrative nociceptive mechanisms, including spinal and higher brain structures mediating pain sensation, a full understanding of itch is lacking. Much evidence exists regarding spinal mechanisms mediating pruritogen induced activity (Patel and Dong, 2010), yet very little is known regarding brain regions involved. Chronic itch is a debilitating condition affecting 1 in 10 people (Steinhoff et al., 2003), thus understanding the mechanisms involved in the experience of itch is of paramount importance. Direct injections of histamine or PAR2 agonists into skin tissue induces obvious scratching behavior in mice, and provide reliable animal itch models that have begun to broaden our understanding of itch mechanisms in mammals.

4.1.3 An integrative system for itch: evidence for cortical modulation

Spinal dorsal horn neurons are activated by pruritogens (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).
Glutamate may be a key neurotransmitter mediating itch and pain induced behaviors. For example, C-fibre simulation activates inhibitory interneurons in the substantia gelatinosa of the dorsal horn, with direct connections onto lamina I neurons (Davidson and Giesler, 2010). A subpopulation of inhibitory interneurons in the dorsal horn of mice, that require Bhlhb5 to survive, were ablated through mutagenesis inhibition of Bhlhb5 expression (Ross et al., 2010). These mutant mice showed exaggerated pruritogen-induced scratching behavior, indicating that scratching can be modulated by disinhibition of 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 pruritogen-induced scratching (Sun and Chen, 2007; Sun et al., 2009).

Histamine dependent and independent types of itch have been found to activate STT neurons (Davidson et al., 2007; Namer et al., 2008), and human imaging studies show both types of itch correspond with activity in the ACC (Ikoma et al., 2006; Yosipovitch et al., 2008; Papoiu et al., 2012). The ACC receives robust projections from the thalamus and is involved in emotional and attentive responses to noxious stimulation (Rainville et al., 1997; Lee et al., 2007b; Zhuo, 2008). 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; 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.
4.1.4 KA receptors may be involved in itch

In the ACC, KA receptor GluK1 subunits mediate small EPSCs (Wu et al., 2005a), which increase in frequency and amplitude in response to high frequency stimulation. Most investigations of ACC excitatory transmission have focused on glutamate mediated AMPA and 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 stimuli (Ko et al., 2005). Specifically, mutant mice with global knockout of the KA receptor GluK1 subunit showed reductions in behavioral responses to acute inflammatory pain induced by formalin injection, but showed no differences in chronic inflammatory pain induced by CFA. The possible contributions of ACC KA receptors to behavioral responses to itch or pain however have not been reported. The following sets of experiments provide a combination of behavioral, pharmacological, electrophysiological, and genetic data that investigated the role of ACC KA receptors in pruritogen-induced scratching. The findings 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.

4.2 Methods
4.2.1 Animals

GluK1−/− mice were a gift from Dr. Stephen F. Heinemann (The Salk Institute, San Diego) and were maintained on a C57BL/6 background; C57BL/6 mice (Charles River, Quebec Canada) were used as controls, as previous observations showed no differences in scratching between C57BL/6 and wild-type littermates. 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.

4.2.2 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 X 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 minutes post application of pruritogens, where we defined one bout of scratching by either hind paw directed towards the nape of the neck as a scratching episode. Behavioral observations were performed blind.
4.2.3 Behavioral acute nociception

In the hotplate test, mice were placed on a standard thermal hotplate with a heated surface (55 °C) (Columbus Instruments, Columbus, OH). The latency for nociceptive responses was recorded with a cut-off time of 30 seconds. The spinal nociceptive tail-flick reflex was evoked by radiant heat (Columbus Instruments, Columbus, OH) applied to the underside of the tail, and latencies were measured with a cut-off time of 10 seconds. All tests were performed blind.

4.2.4 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) as described previously (Wu et al., 2007). 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 4 to 40 rpm over a 5 minute 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 seconds and a 5 minute 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.

4.2.5 Itch inducing stimuli (pruritogens)

Histamine (250 or 18 μg/ 10 μl, dissolved in saline, Sigma-Aldrich), protease-activated receptor-2 (PAR2) agonist peptide, SLIGRL-NH₂ (25 μg / 10 μl, dissolved in saline, Tocris), 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.

4.2.6 Drugs

UBP-302 (Tocris) was dissolved in DMSO to a concentration of 50mM. Further dilutions were made with sterile saline. For ACC microinjections, concentrations of 20 nM, 2 μM, 200 μM, and 3mM were used, and for i.p. injections 50 mg/kg. Cortical vehicle (6% DMSO in sterile saline) injections were used as control. 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.
**4.2.7 ACC cannulae implantation and microinjection**

Bi-lateral cannulas were implanted into the ACC of mice as reported previously (Wu et al., 2005b). Mice were anaesthetized with inhaled 2-3% isoflurane, and the fur above the skull was shaved and disinfected. Mice heads were secured on a stereotaxic frame and 24-gauge guide cannulas were implanted bilaterally into the ACC (0.7 mm anterior to bregma, ± 0.3 mm lateral from the midline, 0.9 mm beneath the surface of the skull). Mice were given two weeks to recover after cannula implantation. Bi-lateral intra-ACC injections were delivered via a 30-gauge injection cannula that was lowered 0.85 mm further into the brain than the guide. The microinjection apparatus consisted of a Hamilton syringe (10 µl) connected to an injector needle (30 gauge) by a thin polyethylene tube and motorized syringe pump. All infusions consisted of 0.5 µl of solution delivered at a rate of 0.05µl/min. Injection sites were confirmed at the end of all experiments and sites outside of the ACC region were excluded from the study.

**4.2.8 Immunohistochemistry**

Immunostaining was performed as described previously (Wu et al., 2008) (Bair et al., 2008; Li et al., 2010). Mice were exposed to histamine, or saline, and 90 min later were heavily anesthetized with isoflurane and perfused with 0.01 mol/l phosphate-buffered saline (PBS; pH 7.4) via the ascending aorta followed by perfusion of 4% paraformaldehyde (PFA) in 0.1 mol/l PB at 4 °C. The brains were removed and post-fixed for 4 hours in 4% PFA, after which brains were placed in vials filled with 30%
sucrose in 0.1 mol/l PB at 4 °C for at least 48 hours, or until the brain fully dropped to the bottom of the jar. Brain sections containing the ACC were cut using a cryostat (Leica) at 30 µm thickness. Briefly, sections were sequentially incubated through the following solutions: (i) a solution of 3% bovine serum albumin (BSA; Sigma, St Louis, USA) and 0.3% Triton X-100 containing anti-c-fos (1:12 000 abcam) primary antibody for 3 days at 4 °C. (ii) Biotin labelled goat anti-rabbit secondary antibody (1:1000 Santa Cruz, CA) for 24 hours at 4 °C, and (iii) Cy3 conjugated streptavidin (1:1000; Santa Cruz, CA, USA) for 2 hours at room temperature. In between each step, sections were rinsed with PBS 3 times for 10 min. Sections were mounted on gelatin coated slides, air-dried, cleared and cover-slipped for observation under a confocal microscope (FV-1000, Olympus, Japan). All slices were scanned and analyzed blind.

4.2.9 Electrophysiology

Thirty min 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., 2005a; Fukushima et al., 2008; Li et al., 2010). Slices were then transferred to a submerged recovery chamber with oxygenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgSO₄, 25 NaHCO₃, 1 NaH₂PO₄, and 10 glucose at room temperature for at least 1 hr. 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 postsynaptic currents (sEPSCs) in histamine, and saline groups 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., 2005a). For frequency facilitation, repetitive stimulation was delivered at 200 Hz (5, 10, or 20 shocks) (Wu et al., 2005a). Evoked excitatory postsynaptic 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 MgCl₂, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na₃-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. Electrophysiological recordings were performed by Dr. Kohei Koga.
4.2.10 Data Analysis

Results are expressed as mean ± SEM. Statistical comparisons were made using unpaired and paired student t-tests, or one-way Analysis of Variance (ANOVA). Significant effects were further tested by the Holm-Sidak test for multiple comparisons. In all cases, $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 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. Data was first collected comparing GluK1−/− mice bred with a C57 background to investigate if GluK1 containing KA receptors may play a role in pruritogen-induced behavior. Adult male GluK1−/− and age matched C57 mice were exposed 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 4.1a). These experiments were repeated 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, \( t = 3.9, P < 0.05 \); Figure 4.1b). These results clearly 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 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., 2009b). Similar to the 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, \( t = 3.6, P < 0.05 \); Figure 4.1c). In addition, the effects of the naturally occurring PAR2 agonist, cowhage were also tested. 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 matched C57 mice (C57: 63 ± 8 bouts, n = 8; GluK1 \( ^{-/-} \): 16 ± 6 bouts, n = 8, \( t = 4.6, P < 0.001 \); Figure 4.1d). These results strongly indicate that GluK1 receptors are involved in histamine and non-histamine induced scratching behavior.
Figure 4.1: 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 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 PAR2 stimulation by SLIGRL-NH2 (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. (*P < 0.05)

4.3.2 Pharmacological inhibition of GluK1-containing KA receptors attenuates scratching

The next set of experiments sought to determine if pharmacological inhibition of KA receptor GluK1 subunits could attenuate itch induced scratching. Adult C57 mice were exposed 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 the knockout data, 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_{(1mg/kg)}: 94 ± 16 bouts, n = 6; UBP-302_{(10mg/kg)}: 84 ± 15 bouts, n = 6; UBP-302_{(50mg/kg)}: 43 ± 7 bouts, n = 9, F_{3,19} = 25.5, P < 0.001; Figure 4.2a). 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, P > 0.05; Figure 4.2b), 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 sec, n = 6; UBP-302: 199 ± 11 sec, n = 6, t = - 5.9, P > 0.05; Figure 4.2c), excluding the possibility that the effects produced by UBP-302 are due to disruptions of motor ability.
**Figure 4.2: 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 (50mg/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 < 0.05).

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

Thus far we have discussed 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, implanted bi-lateral cannulae
were implanted into the ACC of adult C57 mice (Wang et al., 2011) which allowed for microinjections of 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 Figure 4.3a, 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 2μM 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 = 6; UBP-302_{20 nM}: 76 ± 9 bouts, n = 6; UBP-302_{2 μM} 63 ± 14 bouts, n = 6; UBP-302_{200 μM}: 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_{4,26} = 8.24, P < 0.001). In contrast, no differences in response latencies where observed in the hot plate test across all 4 doses of cortical UBP-302 injections, compared with cortical vehicle injections (vehicle: 8.33 ± 0.31 s, n = 4; UBP-302_{20 nM}: 7.22 ± 0.25 s, n = 4; UBP-302_{2 μM} 7.5 ± 0.56 s, n = 4; UBP-302_{200 μM}: 7.67 ± 0.06 s, n = 4; UBP-302_{3 mM}: 7.24 ± 0.44 s, n = 4, F_{4,15} = 1.57, P > 0.05; Figure 4.3b), 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. Experiments were then performed to confirm that the effects of cortical UBP-302 were indeed indicative of cortical KA receptor modulation of scratching, and not due to a general effect of behavior suppression by changes in ACC excitability. C57 mice were exposed to cortical injections of AP-5, an NMDA receptor antagonist, and CNQX, an AMPA and KA receptor antagonist, and the effects on histamine induced scratching were observed. Remarkably, although a one-way ANOVA
showed a significant difference between groups \( F_{2,16} = 4.43, P < 0.05 \), posthoc analysis revealed that ACC injections of AP-5 had no effect on behavioral scratching, whereas significant reductions where induced by CNQX injections (saline: 103 ± 12 bouts, n = 7; AP-5: 105 ± 12 bouts, n = 6; CNQX: 62 ± 9 bouts, n = 6; Figure 4.3c).

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\textsubscript{3 mM}: 51 ± 10 bouts, n = 7; \( t = 5.3, P < 0.001 \); Figure 4.3d). These results clearly demonstrate that inhibition of GluK1-containing KA receptors in the ACC can reduce scratching induced by distinct classes of itching stimuli, and that reduction of excitability within the ACC is insufficient to affect scratching.
Figure 4.3: Pruritogen-induced scratching involves GluK1-containing KA receptors in the 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 4 doses of intra-ACC UBP-302 injections corresponded with 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 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)

4.3.4 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., 2009b). 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). After exposure to intradermal injections of histamine or saline, C57 were perfused and brains were extracted and prepared for immunostaining (see methods 4.2.8). As can be observed in Figure 4.4b, 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 x 10^5 µm², n = 3, t = 11.6, P < 0.001) and layers V/VI (C57: 30 ± 4 cells, n = 3; GluK1−/−: 7 ± 1 cells/1 x 10^5 µm², n = 3, t = 6.3, P < 0.05; Figure 4.4c-d). Saline application induced a small amount of c-Fos expression that was indistinguishable
between groups (Figure 4.4e-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; although please note that ACC slices were collected from C57 wild types and GluK1−/− mutant mice that were bred separately.
Figure 4.4: Pruritogen induced c-Fos expression in the 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)

4.3.5 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 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 postsynaptic currents (sEPSCs) and observed significant increases in frequencies (Histamine: 11.2 ± 1.8, n = 11; Saline: 5.6 ± 2.0, n = 11, t = 2.08, P < 0.05; Figure 4.5a-c) and amplitudes (Histamine: 9.3 ± 1.0 Hz, n = 11; Saline: 7.1 ± 0.3, n = 11, t = 2.11, P < 0.05; Fig. 4.5a-c) of sEPSCs. Taken together, these results suggest
that histamine-induced scratching corresponds with increases spontaneous excitatory transmissions in the ACC.

Figure 4.5: Itching stimuli corresponds with alterations of sEPSCs in ACC pyramidal neurons. a) Representative sample traces of spontaneous excitatory postsynaptic currents (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). ** Data for this figure was collected by Dr. Kohei Koga
4.3.6 Itching stimulation does not recruit postsynaptic KA receptor activity in ACC pyramidal neurons

Next, we wanted to determine whether postsynaptic KA receptor function is altered in mice exposed to histamine or saline, and thus recorded postsynaptic 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., 2005a). The single pulse stimulation yielded AMPA/KA mediated EPSCs in ACC neurons from mice exposed to saline or histamine (Figure 4.6a). 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, P > 0.05; Fig.4. 6b). This suggests that histamine does not alter postsynaptic 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 (Figure 4.6c), with similar resulting amplitudes between both groups (Figure 4.6d). 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 change postsynaptic KA mediated synaptic efficacy in ACC pyramidal neurons.
Figure 4.6: Itching stimuli does not enhance postsynaptic KA receptor activity in 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 AMPA/KA mediated 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) Input-output relationships of KA receptor mediated EPSCs were also similar.  ** Data for this figure was collected by Dr. Kohei Koga.

4.3.7 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 evoked excitatory postsynaptic potentials (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\textsubscript{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 % of baseline; post UBP-302: 81.6 ± 1.2 % of baseline, n = 8, t = 2.65, P < 0.05; Figure 4.7a 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, P > 0.05; Figure 4.7a and c). 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, P > 0.05; Figure 4.7c and c), indicating that the EPSP attenuating effect of UBP-302 is being mediated via GABAergic transmission. Taken together, these observations suggest that pruritogen-induced KA receptor GluK1 subunit activity modulates inhibitory transmission in the ACC.
Figure a: Graph showing the effect of Saline and Histamine injected alone or in combination with Baseline and UB-302 on the Amplitude of eEPSP (%). The x-axis represents time in minutes, ranging from 0 to 25, while the y-axis represents the amplitude in percentage. The data points are marked with error bars.

Figure b: Graph showing the effect of Saline and Histamine injected alone or in combination with Baseline and UB-302 with added Picrotoxin. The data points are marked with error bars.

Figure c: Bar graph comparing the Amplitude of eEPSP (%) before and after UB-302 treatment. The bar charts show the comparison between Saline, Saline + Picrotoxin, Histamine, and Histamine + Picrotoxin treatments. The x-axis represents the different treatments, and the y-axis represents the amplitude in percentage.
Figure 4.7: Pruritogen-induced 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 postsynaptic 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<sub>A</sub> 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) Remarkably, in the presence of picrotoxin (100 µM), the attenuating effect of UBP-302 was blocked. c) Summarized data of the amplitudes of eEPSPs in saline and histamine group with or without picrotoxin. (*P < 0.05) ** Data for this figure was collected by Dr. Kohei Koga

4.3.8 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, behavioral response latencies were measure in response 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 sec, n = 6; GluK1 +/-: 5.1 ± 0.24 sec, n = 5, t = 1.8, P > 0.05; Figure 4.8a) and tail flick tests (C57: 6.2 ± 0.3 sec, n = 6; GluK1 +/-: 5.9 ± 0.6 sec, n = 5, t = 0.3, P > 0.05; Figure 4.8b), indicating that KA receptor GluK1 subunits do not mediate acute physiological pain. In contrast, it has been previously shown that GluK1 +/- mice display deficits in formalin (5%) induced nociceptive licking behavior (Ko et al., 2005), and that formalin (5%) injection is accompanied by robust c-Fos expression in ACC neurons (Wei et al., 2001). Nociceptive responses were thus next compared 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 (3mM), and adult naïve C57 mice. In accordance to previous reports, GluK1 +/- mice exhibited reduced nociceptive behavior compared to C57 mice, and remarkably, this reduction was replicated in C57 mice exposed to ACC microinjections of UBP-302 (Figure 4.8c). A repeated measures two-way ANOVA showed a significant interaction between group and time ($F_{(2,15)} = 10.28, P < 0.001$; Figure 4.8d), whereby GluK1 +/- mice and C57 mice with ACC injections of UBP-302 displayed similar nociceptive behavior as C57 mice during Phase 1 (first 10 min) of the formalin test (C57: 59.4 ± 11.9 sec, n = 6; GluK1 +/- 58.9 ± 9.6 sec, n = 6, UBP-302: 61.7 ± 7.3 sec, n = 6), but showed a robust reduction in nociceptive
behavior during phase two (10 – 60 min) (C57: 422 ± 66.2 sec, n = 6; GluK1 \(^{-/-}\) 180.5 ± 12.9 sec, n = 6, UBP-302: 189.1 ± 21.5 sec, n = 6; Figure 4.8d), with similar attenuation being observed in GluK1 \(^{-/-}\) and UBP-302 treated C57 mice. 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.
Figure 4.8: 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. (*P < 0.001)

4.4 Discussion

This study shows that cortical GluK1-containing KA receptors modulate behavioral responses to itch and inflammatory pain. The results demonstrate that genetic deletion or pharmacological inhibition of GluK1 subunits attenuates scratching induced by histamine and non-histamine dependent pruritogens. Furthermore, inhibition of GluK1 specifically within the ACC also reduces scratching, and pruritogen-induced neuronal activity in the ACC is reduced in GluK1 

\[ \text{Δ/Δ} \] 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, results show that pharmacological inhibition of GluK1 subunit activity within the ACC also reduces behavioral responses to inflammatory pain.

4.4.1 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. These sets of
experiments have presented evidence of cortical contribution to scratching in mice by showing that intra-ACC injections of UBP-302 reduce pruritogen-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., 2007b), 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, these results show that scratching behavior corresponded with ACC activity, and scratching has been previously observed to correspond with motor cortex activity (Sirota et al., 2006). Likewise, 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).

4.4.2 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 show some itch specific spinal mechanisms (Schmelz et al., 1997; Sun
and Chen, 2007), others observed interactions between both experiences (Ikoma et al., 2006; Yosipovitch et al., 2008; Davidson and Giesler, 2010), suggesting they share similar mechanisms. The present data indicates 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 and itch may have distinct spinal mechanisms (Patel and Dong, 2010), these results 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, 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 the pharmacological ACC microinjection experiments provide direct evidence for the requirement of cortical GluK1 in pruritogen-induced scratching and pain-induced licking, possible spinal and peripheral contributions of KA receptors in these behaviors cannot be ruled out (Kerchner et al., 2001).
4.4.3 Cortical kainate GluK1 receptor mechanisms

Cortical inhibition of GluK1-containing KA receptors robustly reduced pruritogen-induced scratching and enhancements of glutamatergic transmission in the ACC. Remarkably, cortical inhibition of GluK1-containing KA receptors did not affect acute nociceptive behavior, but reduced inflammatory pain induced licking. 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. Previous studies show that pyramidal neurons of layers II/III in the ACC express postsynaptic KA receptors containing GluK1 subunits (Wu et al., 2005a), and these receptor subunits mediate a small component of the postsynaptic excitatory current. In the present study however, itching stimuli did not enhance postsynaptic KA receptor activity. Taken together, the data suggests a possible cortical mechanism whereby itching stimuli induces glutamatergic release at ACC synapses, activating postsynaptic AMPA receptors and presynaptic GluK1-containing KA receptors on inhibitory neurons, which in turn modulate GABAergic release (Figure 4.9). Indeed, these experiments showed that pruritogen application enhanced depression of postsynaptic excitatory potentials by UBP-302, indicating an increase in GluK1-containing KA receptor activity. Furthermore, this enhanced susceptibility was completely blocked by GABA\(_A\) antagonism, indicating that GluK1 subunits induced modulation by acting through GABAergic transmission. It has been 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 presynaptic KA receptors can modulate GABAergic inhibition in the mammalian brain (Lerma, 2003). The present data shows that cortical GluK1 activity is involved in pruritogen-induced scratching, and suggests that GluK1 modulation of evoked GABAergic 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.
Figure 4.9: Pruritogen dependent KA receptor modulation of evoked GABAergic transmission in the 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 presynaptic glutamate (Glu) release activates postsynaptic AMPA receptors located on pyramidal neurons thereby enhancing the postsynaptic response. Simultaneously, presynaptic Glu can activate presynaptic kainate receptors (KAR) located on inhibitory neurons, thereby modulating GABA release and affecting the attenuation of the postsynaptic response.
CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS
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5.1 Integrated discussion

A major theme recurrent throughout this thesis is that neural activity within the brain underlies complex behavior and mental states, and is responsible for our ability to perceive, act, feel, and remember. This thesis focused on bridging *in vitro* and *in vivo* observations to determine the neural substrates for ACC mediated behavior. This critical step allows for the identification of the neural substrates responsible for complex human experiences. Experiments presented in this thesis combined behavioral, pharmacological, genetic, biochemical, and electrophysiological observations to assess cortical mechanisms responsible for fear learning and sensation related behaviors. Chapter two focused on cortical LTP-like mechanisms, and investigated whether they could mediate fear learning. These sets of experiments provided *in vivo* evidence that fear learning induces GluN2B mediated activity that drives cortical AMPA receptor insertion. Subsequent investigations, Chapter 3, presented direct evidence that forebrain CREB-mediated transcription contributes to behavioral allodynia in animal models of chronic inflammatory or neuropathic pain. Lastly, Chapter 4 showed that pruritogen-induced scratching corresponds with enhanced excitatory transmission in the ACC through KA receptor modulation of inhibitory circuitry. Collectively, the data presented in this thesis shows evidence that manifestations of behaviors related to ACC activity can be observed at the molecular level within the ACC. These findings indicate
that molecular mechanisms involved in ACC synaptic activity present a good target for translational research into pathological conditions observed to correspond with emotional and attentional components.

5.2 LTP-like mechanisms within the ACC mediate trace fear learning

Although the cortex has been extensively studied in long-term memory storage, less emphasis has been placed on immediate cortical contributions to fear memory formation. The experiments presented in Chapter two provide strong evidence that rapid cortical AMPA receptor plasticity mediates fear learning and memory. Data covered included evidence that the ACC shows a rapid 20% upregulation of membrane AMPA receptor GluA1 subunits that is evident immediately after conditioning. Inhibition of NMDA receptor GluN2B subunits during training prevented the upregulation, and disrupted trace fear memory retrieval 48 h later. In vivo experiments and ex vivo electrophysiological recordings implicated CP-AMPARs as mediators of synaptic strengthening processes responsible for fear memory formation. Chapter 2 results thus indicate that trace fear learning is mediated through rapid GluN2B dependent trafficking of CP-AMPARs. Although various publications indicate that NMDA receptors in several brain regions are involved in fear memory, including the amygdala (Zhang et al., 2008b), hippocampus (Huerta et al., 2000; Zhang et al., 2008a), and forebrain (Cao et al., 2007), studies had yet to provide evidence of the learning related downstream target. More importantly, several important studies have questioned the requirement of NMDA GluN2B receptors in hippocampal LTP and learning (Huang and Malenka, 1993;
Liu et al., 2004). Furthermore, although reports have shown that NMDA receptor GluN2B subunit activity is critical for ACC LTP (Toyoda et al., 2005), that AMPA receptor insertion corresponds to potentiation of excitatory synaptic transmission (Gu et al., 1996; Boehm et al., 2006; Clem and Barth, 2006), and that NMDA activity can induce AMPA receptor trafficking in vitro (Shi et al., 1999), no study had shown in vivo evidence that learning induces GluN2B mediated AMPA receptor insertion. This important proof of concept is critical for our understanding of the molecular mechanisms behind learning and memory. These findings link important in vitro mechanisms of ACC LTP with in vivo learning by demonstrating that in the ACC, key LTP players are also critical for memory formation.

5.2.1 Limitations of Chapter 2 experiments

AMPA receptor GluA1 subunits protein levels were quantified through western blot analysis of membrane fractions. These fractions were derived through a series of steps aimed at separating nuclear, cytosolic, and membrane components of ACC tissue samples. Such manipulations may allow for some endoplasmic reticulum (ER) components to be included as it is rich in membrane and AMPA receptors are assembled in the ER (Shepherd and Huganir, 2007), and thus some quantified GluA1 protein may not be in the synapse. It is unlikely however that much of the quantified protein was located within the ER, as electrophysiological recordings showed a trace fear induced increase in sensitivity to NASPM, likely reflecting increases in functional GluA2 lacking AMPA receptors at synapses (Clem and Huganir, 2010).
A major caveat to the electrophysiological recordings in Chapter 2 (and also Chapter 4) is that acute slices do not allow for input specificity. Although this is certainly true, our findings go well beyond *in vitro* electrophysiology. Indeed, our main findings are broadly replicated across our biochemical, behavioral, and electrophysiological data. Moreover, various impactful publications have used acute brain slices from mice to detect experience induced changes in excitatory transmission (Clem and Barth, 2006; Kessels and Malinow, 2009; Clem and Huganir, 2010). Conversely, acute brain slices may contain axonal projections from various brain areas, and thus it is possible that some evoked EPSCs could be contaminated by fibers arising from brain areas which are not recruited during trace fear learning.

### 5.3 Injury induced CREB-mediated transcription mediates chronic pain behavior

Chapter 3 showed that genetic enhancement of CREB-mediated transcription selectively in forebrain areas enhanced behavioral responses to non-noxious stimuli after nerve injury or chronic inflammation (CFA model). These experiments provide direct evidence that cortical CREB-mediated transcription contributes to behavioral allodynia in animal models of chronic inflammatory or neuropathic pain. Previous reports indicated that CREB is activated by injury and can be induced by in spinal dorsal horn neurons and the ACC. However no report showed direct evidence for the role of activated CREB in injury-related behavioral sensitization. Baseline mechanical and acute pain responses to noxious thermal stimuli were not affected by genetic CREB
enhancement, supporting previous reports that CREB exerts its effects in an activity dependent manner (Deisseroth et al., 1996).

5.3.1 Limitations of Chapter 3 experiments

An obvious possibility is that enhancement of CREB mediated transcription in other forebrain areas, other than the ACC, are mediating increases of injury induced behavioral sensitization. Indeed, the central nucleus of the amygdala receives spinal nociceptive input (Sarhan et al., 2005) and displays plasticity in chronic pain conditions (Neugebauer et al., 2004). The decreased behavioral response to acute inflammatory pain was unexpected and may reflect additional effects of CREB overexpression. For example, licking is not always beneficial, and may even enhance the inflammatory response; as CREB overexpressed mice show enhanced fear memory (Suzuki et al., 2011), it is possible that these transgenic mice are more adept at identifying a fruitless behavior.

5.4 Cortical GluK1 KA receptors modulate scratching behavior

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. The final set of experiments in this thesis focused on cortical itch mechanisms. These findings show evidence of cortical
modulation of pruritogen-induced scratching behavior. The data combined genetic, behavioral, and pharmacological approaches to show that cortical GluK1-containing KA receptors are involved in scratching induced by histamine and non-histamine dependent itching stimuli. Furthermore, electrophysiological recordings showed that scratching corresponds with enhanced excitatory transmission in the ACC through KA receptor modulation of inhibitory circuitry. These studies thus identified a novel role for cortical KA receptors by determining the in vitro correlates to in vivo observations; that is by linking the effects of genetic and pharmacological inhibition of GluK1 KA receptors on pruritogen-induced scratching, to pruritogen-induced changes in pharmacologically identified KA mediated excitatory transmission. These results reveal a new role of the cortex in pruritogen-induced scratching.

5.4.2 Limitations of Chapter 4 experiments

Although the present data focused on cortical mechanisms, and through intra-ACC injections directly observed the necessity of GluK1 ACC activity in pruritogen-induced scratching, spinal mechanisms cannot be excluded. Indeed, GluK1 containing KA receptors play a role in glutamatergic synaptic transmission in the dorsal horn, and have been observed to regulate synaptic transmission at excitatory and inhibitory synapses (Kerchner et al., 2001; Lerma, 2003). Nevertheless, electrophysiological recordings directly from ACC neurons show evidence of pruritogen induced changes in GluK1activity, and thus the data clearly supports that GluK1 activity in the ACC is at least in part responsible for pruritogen-induced scratching.
Pharmacological interventions can be difficult to interpret, and caution should always be taken when generalizing behavioral observations with complex brain mediated experiences. Although we cannot exclude the possibility of non-specific effects of UBP-302 on behavior, we show that cortical injections of UBP-302 do not affect acute nociceptive behavior across 4 different doses, indicating that cortical injections of UBP-302 do not have a general effect of suppressing sensation related behaviors. It is important to note however that formalin and histamine have also been observed to induce both pain and itch sensations, and thus some of our findings may not be specific to one sensation or the other. The ACC receives robust projections from thalamic nuclei, and thus receives robust nociceptive input. To determine the specificity of KA receptors in pruritogen-induced scratching, and not just a general role for the ACC in nocifensive behavior, microinjections of AP-5 and a mild dose of CNQX were employed; in both cases, the local excitability would have been attenuated. Cortical injections of AP5 did not affect scratching behavior in mice compared to vehicle injections, showing that a reduction in local excitability in the ACC is insufficient to modulate pruritogen-induced scratching. In contrast, CNQX did significantly attenuate scratching, which is in accordance with our present findings for a couple of reasons. First, CNQX is an AMPA and kainate receptor antagonist, and thus conceivably reduced GluK1 receptor activity within the ACC. Second, scratching corresponds with glutamatergic mediated excitatory currents in the ACC, thus attenuation of AMPA receptor mediated glutamatergic transmission should also decrease scratching behavior.
Although it is impossible to fully dissociate sensation from behavior in an animal models, pure involvement of the ACC in scratching motor responses and not the sensation of itch however seem unlikely. For example, electrical stimulation (Tang et al., 2005) or chemical activation (Johansen and Fields, 2004) of the ACC do not produce 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 Elizabethanean collars could be used to prevent mice from successfully scratching, mice still attempt to scratch and thus still perform the same hindpaw behavioral movements.

5.4.3 Conclusions and future directions

The studies presented in this thesis highlight cortical mechanisms mediating fear learning, chronic pain, and pruritogen-induced behaviors. Although distinct mechanisms were the focus of particular experiments (i.e. NMDA and AMPA receptors in fear learning; transcription factors for chronic pain; and KA receptors in scratching), it is obvious they all involve excitatory transmission in the ACC. It is therefore likely that there is overlap between the cortical mechanisms responsible for fear, pain, and scratching induced behaviors. Such overlap may provide an avenue through which to affect specific behaviors. For example, attention to the CS during fear learning is critical for proper memory formation, as visual distraction corresponds with reductions in memory recall and conditioning induced ACC activity as evidence by c-Fos staining (Han et al., 2003). Similarly, mice with chronic pain show deficits if trace fear learning
(Zhao et al., 2006). It would therefore be of interest to see if under chronic pain conditions, AMPA receptor upregulation has already occurred, and subsequent enhancement is impaired. Such possible saturation of AMPA receptor trafficking may be a possible mechanism through which chronic pain induced impairments in fear learning are mediated. In a similar fashion, it would be of interest to see if pruritogen-induced ACC activity reduces trace fear learning, or if induction of fear can interfere with scratching.

A major challenge in the analysis of animal models is the interpretation of behavior as significant of a particular phenomenon, like ‘itch’. Indeed, deductions are often made about perception of input sensation based on motor output behavior. Behavioral assays are therefore developed to attempt to dissociate spontaneous ‘feelings’, from stimulus evoked responses. Such an assay is the conditioned place avoidance paradigm (CPA), whereby animals can be trained to associate a chamber with a negative or a neutral sensation (Johansen et al., 2001). If pruritogen-induced ACC activity reflects the affective component of itch, then a robust avoidance of the pruritogen paired chamber should be detectable. If however, pruritogen-induced ACC activity reflects mostly motor components of scratching behavior, then the associative memory of the paired chamber should be weak.

CREB has been implicated in various events that are known to correspond with changes in postsynaptic receptors, including fear learning (Josselyn et al., 2001; Han et al., 2009; Suzuki et al., 2011) and drug addiction (McClung and Nestler, 2007). Two follow up experiments are thus easily envisioned. First, given the present finding that trace fear learning requires GluN2B mediated cortical upregulation of AMPA receptor
GluA1 subunits, forebrain enhancements of CREB-mediated transcription should result in enhanced GluA1 upregulation, and increases in fear memory recall. Indeed it would be interesting to determine if enhancements in CREB can enhance acquisition, that is establish CS-US associations with fewer trials or massed trials, or even with weaker US stimulation or longer trace periods. Observations from CREB overexpression in the amygdala and subsequent enhancements in fear memory certainly suggest these possibilities (Josselyn et al., 2001).

In summary, this thesis has presented corresponding *in vitro* and *in vivo* data providing evidence of molecular mechanisms in the ACC that are involved in fear learning and nociceptive related behaviors. The results indicate that manifestations of behavior related to ACC activity can be observed at the molecular level, and suggests that targeting molecular mechanisms involved in ACC synaptic activity present a good opportunity for translational research into pathological conditions that involve emotional and attentional components of fear and nociception, such as PTSD and chronic pain.
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