

REVIEW

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# Cortical plasticity as a new endpoint measurement for chronic pain

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## Abstract

Animal models of chronic pain are widely used to investigate basic mechanisms of chronic pain and to evaluate potential novel drugs for treating chronic pain. Among the different criteria used to measure chronic pain, behavioral responses are commonly used as the end point measurements. However, not all chronic pain conditions can be easily measured by behavioral responses such as the headache, phantom pain and pain related to spinal cord injury. Here I propose that cortical indexes, that indicate neuronal plastic changes in pain-related cortical areas, can be used as endpoint measurements for chronic pain. Such cortical indexes are not only useful for those chronic pain conditions where a suitable animal model is lacking, but also serve as additional screening methods for potential drugs to treat chronic pain in humans. These cortical indexes are activity-dependent immediate early genes, electrophysiological identified plastic changes and biochemical assays of signaling proteins. It can be used to evaluate novel analgesic compounds that may act at peripheral or spinal sites. I hope that these new cortical endpoint measurements will facilitate our search for new, and more effective, pain medicines, and help to reduce false lead drug targets.

## Introduction

Pain has at least two different major forms: acute pain that is short-lasting, and often serves as protective and learning signals. It is also called physiological pain in part due to its important physiological functions. The second form is chronic pain that is long-lasting pain caused by injury to tissue or nerve systems. Although it also informs animal or patients the location of the injured area, the long-term component of chronic pain is not physiological critical, and it causes cognitive impairment, emotional sufferings, loss of sleep and mood disorders. Thus, it is also called pathological pain.

Due to the lack of understanding of cellular and molecular mechanisms of pain mechanisms; the black box experimental approach for studying pain has been widely used by basic researchers and drug developers (Figure 1). Among many different experimental approaches, behavioral reflexes in response to noxious stimuli, or allodynic stimulation after tissue or nerve injury are widely used for the investigation of potential analgesic effects of new drugs, and often proposed as

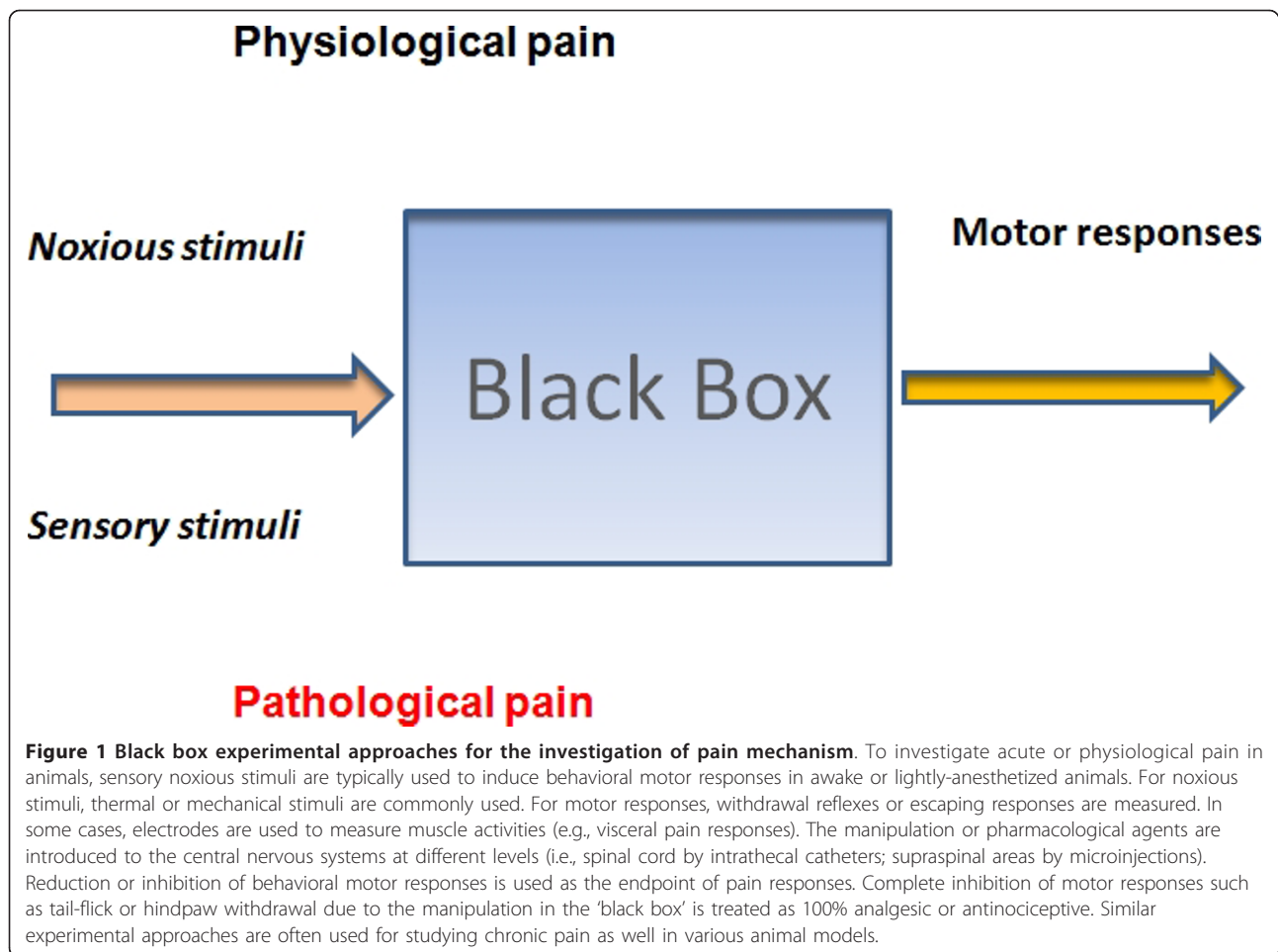
the endpoint of pain indexes. The use of neurobiological approaches is mainly targeted for the investigation of basic mechanisms of pain transmission, modulation and plasticity; and has not been used or proposed as the endpoint measurement of pain.

The progress made in human brain imaging has significantly improved our understanding of chronic pain. Brain imaging in conscious humans allow us to evaluate roles of various cortical areas in pain, and brain activation by painful stimuli can be evaluated and compared with patient's psychological reports of pain and emotional feelings [1]. Recently such techniques have also been successfully used to measure psychological pain or empathy of pain [2,3]. And the measurement of reflexive responses in such human studies is not needed. Can we achieve similar aims in animal studies of chronic pain? In this review, I would like to propose that neurobiological indexes obtained from pain-related cortical neurons can be used as new cortical endpoints of pain measurements in animal models of chronic pain. The use of such new endpoints will allow us to study certain types of chronic pain that is difficult to be investigated in animal models due to the lack of motor responses. They are: headaches, seizure induced pain, phantom pain, spinal injury induced pain and chronic back pain.

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Furthermore, these new endpoints provide mechanism based measurements of chronic pain in animal models, and could be used as better assays for screening potential new drugs for treating chronic pain. It is important to point out that I am not proposing to use those new endpoints to replace the existing behavioral models of chronic pain such as measuring threshold in hyperalgesia and behavioral responses to allodynic stimulation in chronic pain conditions. The combination of these cortical and behavioral endpoints can provide excellent pain endpoint measurements with solid basic neurobiological mechanisms. The new cortical endpoints can be also used effectively for basic investigation of pain mechanisms at peripheral tissue/nerve, spinal cord, and subcortical areas.

#### **Problems that we are facing: drug discovery and translational medical researches**

While animal models of chronic pain have greatly facilitated our understanding of basic pain mechanisms, there are still many major problems that cannot be solved using animal behavioral models. Many forms

of clinical pain cannot be mimicked in animal models. For example, it is very difficult to measure 'phantom' pain in amputated animals; and it is impossible to measure nociceptive withdrawal responses in animals with spinal cord injuries. Mimicking headaches and chronic back pain in animals is also proved to be extremely difficult, since there is not a clear behavioral motor response related to such pain that can be measured. Furthermore, even in many pain conditions that the measurement in animal behavioral responses to pain are possible, the pain index is often affected by unwanted side effects on motor or pre-motor functions. For example, in case of animal models of chemotherapy induced neuropathic pain, the same chemicals also caused injury to motor neurons/nerves. Defects in motor functions directly affect the measurement of nociceptive responses, if only motor responses are used as pain index such as withdrawal thresholds or latencies. Finally, most of potential analgesic drugs also act on motor neurons non-selectively. There is great need to develop additional non-behavioral measurements for chronic pain.

### Human imaging provides direct evidence for the roles of cortex in chronic pain

Unlike animal experiments, human brain imaging using functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have greatly increased basic neuronal mechanisms for chronic pain, especially at the brain areas. The imaging in conscious patients provides the unique opportunity to investigate if any abnormal neuronal activities may be related to chronic pain in patients [4-7]. Progress made in human imaging of chronic pain is very impressive. Between year 2000 and now, there is an average of 300 papers published per year when the searching key word 'brain imaging', 'pain' and 'human' were used. To deal with this huge amount of imaging data, some mega-analyses have been performed recently [5,8]. Among different cortical regions, there are five major cortical areas that are consistently responding to pain. They are: anterior cingulate cortex (ACC), insular cortex (IC), primary somatosensory cortex (S1), secondary somatosensory cortex (S2) and prefrontal cortex (PFC) (Table 1). Among them, the ACC is found to be the most reliable area to be activated by different noxious or painful stimuli. In addition to the ACC, the IC is also commonly activated by different painful stimuli [9,10,8] for review].

### Classical pain endpoints —behavioral models of chronic pain

In basic research area, behavioral studies have been commonly used for measuring endpoint of chronic pain. Most of pharmaceutical drug discovery is focused on two major areas: target proteins and the behavioral

endpoints, at least to my knowledge. The behavioral evaluations in animal models of chronic pain are used as the end point for preclinical studies. Despite many limitations of behavioral tests (any pre-motor or motor side effects of drugs or gene mutant can easily affect behavioral responses in freely moving conditions), these 'analgesic' indexes are commonly used, because it is economic, fast, and easily understood by laypersons such as private investors.

The failure to provide consistent non-behavioral endpoints for chronic pain also complicates drug discovery; and too many potential lead drugs work well in animal models of chronic pain. In any pain-related conferences held in recent years, you often hear much more positive drug target proteins than negative ones. Furthermore, the failure to require solid basic scientific evidence by the drug regulator encourages the cheap- and short-cut experimental approaches used by private investors and drug developers. Furthermore, many of these 'analgesic drugs' failed to be translated into clinical drugs, in part due to the fact that only behavioral endpoints were used in many of these preclinical studies.

### Clinical image studies of those unstudied chronic pain

It is easy to tell humans are different from animals in term of reporting pain, although humans complain about pain for just for pain. Previous studies reported that animal vocalization activities may be used to measure pain or especially spontaneous pain [11-13]. However, these indexes prove to be difficult to be used, since animals also generate vocalization activities under other

**Table 1 Cortical areas that are implicated in chronic pain conditions by human brain imaging fMRI that are difficult to be investigated by traditional animal pain models**

Cortical area	Clinical pain conditions	Major discovery
ACC	Migraine/headache Phantom pain SCI induced pain Chronic back pain	Altered excitatory transmission Related to increased imaged activity Increased activity with imaged pain Increased activity
IC	Migraine/headache Chronic back pain Hypothetically induced pain	Altered excitatory transmission Increased imaged activity Increased imaged activity
PFC	Migraine/headache Phantom pain SCI induced pain Chronic back pain Hypothetically induced pain	Altered brain metabolism Related to increased imaged activity Increased activity with imaged pain Correlated with spontaneous pain; reduced gray matter density Increased imaged activity
S1, S2	Migraine/headache Phantom pain SCI induced pain	Altered sensory transmission Correlation of cortical reorganization Correlated with cortical reorganization

**Abbreviations:**

ACC: anterior cingulate cortex.

IC: insular cortex.

PFC: prefrontal cortex.

S1: primary somatosensory cortex.

S2: Secondary somatosensory cortex.

normal physiological conditions such as sex mating [11]. Recent human studies using brain imaging have provided key evidence for the involvement of central nervous systems in several chronic pain conditions that cannot be mimicked in animal models. These include headache, phantom pain, back pain, pain related to spinal cord injury, and patient with functional bowel disorder [14,15]. For example, Willoch et al (2000) reported that in human amputees the activity increased in the ACC and posterior cingulate as well as thalamus is correlated with pain reported in phantom limb, while the activity in the supplementary motor cortex and primary sensorimotor cortex is related to the phantom limb movement [16]. Apkarian et al (2004) reported that chronic back pain is associated with decreased prefrontal and thalamic gray matter density [6], and altered neural activity in the PFC [17].

Recent studies of neural correlates of social exclusion by neuroimaging study found that the ACC is more active during exclusion rather than during inclusion [2], suggesting that psychological rejection in social exclusion also triggers pain-related cortical areas. In case of patients with spinal cord injury, it has been reported that the magnitude of activation in the perigenual ACC and right dorsolateral PFC was significantly correlated with absolute increases in pain intensity triggered by movement imagery [18]. Similar findings have also been reported in patients with chronic low back pain [19]. These human studies are in consistent with previous anatomic studies in animals that somatosensory cortex reorganized itself after spinal cord injury [20]. Cumulative human studies data consistently indicate that cortical activity plays an important role in chronic pain. In many cases, changes in cortical activities without any peripheral stimuli (including spontaneous pain conditions) are sufficient to produce pain in patients.

### A proposal for using cortical plasticity as the endpoint measurements for chronic pain

Here I would like to propose the use of cortical plasticity as an endpoint measurement for chronic pain, including biochemical, electrophysiological and imaging measurements (Table 2). Such non-behavioral measurements can also be used to measure cortical responses in animal models that behavioral tests are applicable such as animal models of inflammatory and neuropathic pain. In cases of types of pain where behavioral measurement are impossible, these cortical markers can be used to evaluate pain. Cortical long-term changes can be used as markers for measuring drugs' effects in animal models of chronic pain. Drugs that act at the level of periphery or spinal cord may reduce or inhibit such activation in the cortex if they are potential analgesic for chronic pain.

**Table 2 Proposed cortical markers for measuring pain endpoints in chronic pain**

Category	Measure index
<i>Immediate early gene</i>	Activation of c-Fos; Egr1; pCREB
<i>Cortical potentiation</i>	Enhanced AMPA receptor mediated EPSCs
<i>Presynaptic enhancement</i>	
<i>Paired-pulse facilitation (PPF)</i>	PPF ratio
<i>mEPSCs</i>	Frequency Amplitude
<i>Postsynaptic potentiation</i>	
<i>AMPA receptor</i>	Inward rectification
<i>NMDA NR2B receptor</i>	Enhanced NR2B sensitive EPSCs
<i>PKMζ inhibition</i>	More sensitive to ZIP inhibition
<i>Biochemistry</i>	
<i>AMPA receptor GluR1</i>	Increased membrane bind AMPA receptor
<i>p-AMPA receptor PKMζ</i>	Increased phosphorylation at PKA site Enhanced pPKMζ
<i>In vivo field LTP</i>	Injury induced LTP
<i>Structural changes</i>	Outgrow of neuronal dendrites and increased spine density
<i>Brain imaging &amp; in vivo whole-cell</i>	Increased spike responses to non-noxious stimuli Enhanced cortical activities before and/or after peripheral stimuli

### 1. Activity-dependent immediate early gene (IEG)

Activity-dependent immediate early genes are known to be activated by neuronal activity in the central nervous system. Peripheral noxious stimuli or injury triggered activation of c-Fos and phosphorylation of cAMP response element binding protein (pCREB) in the spinal cord dorsal horn neurons [21,8]. Such activation in the spinal cord dorsal horn neurons can be inhibited or blocked by drugs or inhibited behavioral sensitization. It is believed that these activity-activated immediate early genes may contribute to long-term plastic changes in spinal sensory synapses, and thus contribute to behavioral sensitization. It is expected that analgesics that acts at the level of spinal cord or periphery for the treatment of chronic pain should at least partially reduce activation of IEGs triggered by the injury.

Activation of IEGs is not limited at the spinal cord level. Along the somatosensory pathways, activation of IEGs have also been reported in the thalamus, and related cortical areas. Among several pain-related

cortical areas, activation of IEGs in cortical neurons after the injury has been reported in the ACC and IC. Using *c-Fos*, NGFI-A (also called *Egr1*, *zif268*), and pCREB, neurons in the ACC are known to be activated by peripheral noxious stimuli, injury or amputation [22-24]. Wei et al (1999) reported that digit amputations in adult rats triggered rapid activation of *Fos*, *Egr1* and CREB in the ACC neurons, especially pyramidal cells located in the layer II-III (Figure 2). Activation of IEGs is bilateral. It persists for two days [23]. Similar activation of IEGs in the ACC has been reported after tissue inflammation, nerve injury and visceral pain [8].

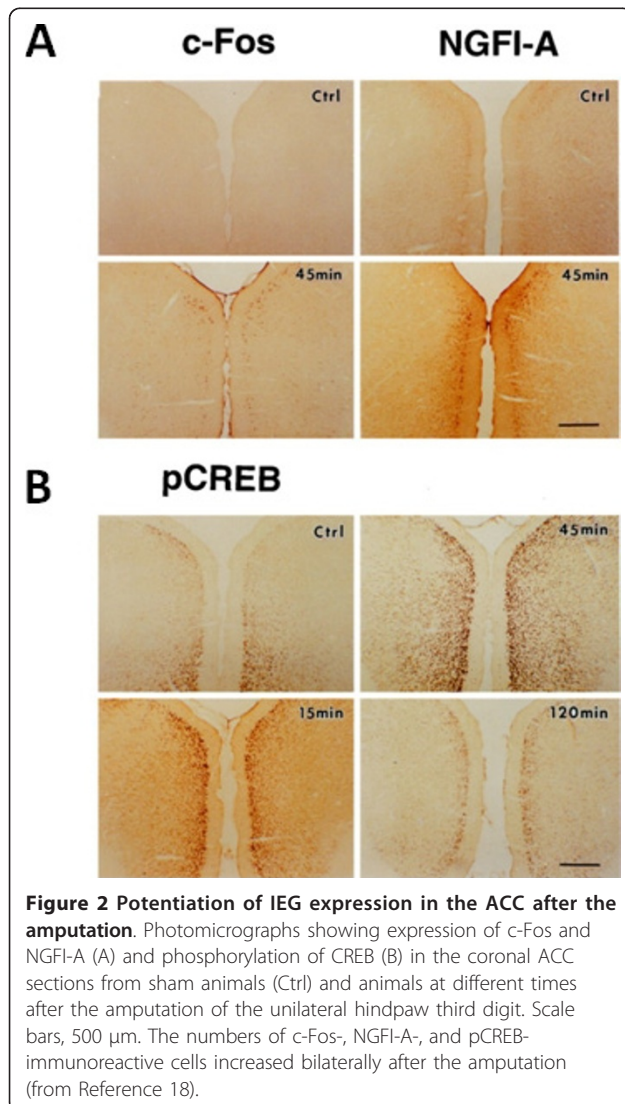
Using gene knockout mice, it has been shown that the amount of IEG activation may be related to behavioral pain phenotypes. In adenylyl cyclase subtype 1 (AC1) or AC1 and AC8 knockout mice, it has been shown that chronic inflammatory pain and neuropathic pain were

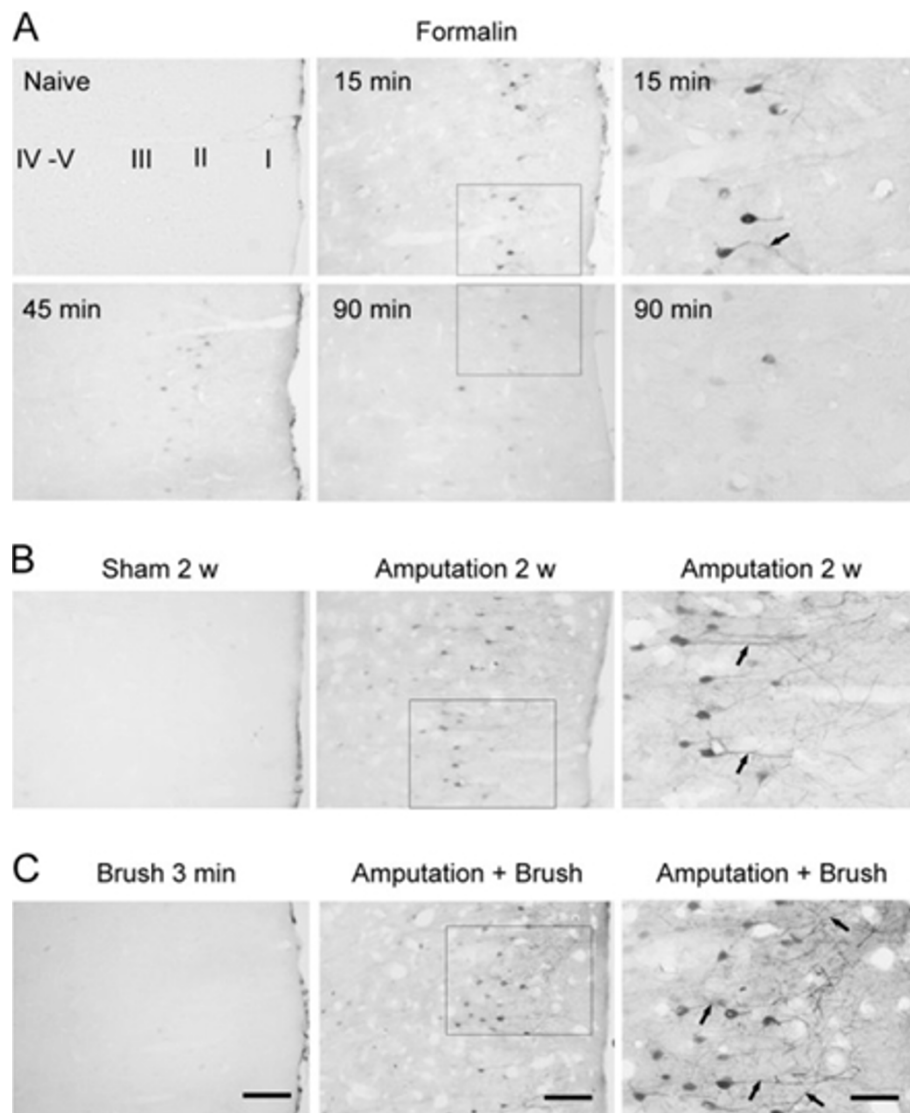
significantly reduced. Consistently, pCREB immunoreactivity induced by hindpaw inflammation was also reduced in AC1, AC8, or AC1&8 double knockout (DKO) mice. Activation of IEGs is also noted in other cortical areas such as IC [25]. Similar to the ACC, the amount of gene expression is likely also related to behavioral pain phenotypes. Interestingly, fear condition, a form of emotional learning, also triggered IEGs in cortical neurons [26]. Activation of Ca<sup>2+</sup>-calmodulin-dependent protein kinase IV (CaMKIV) is functionally important for activation of IEGs as well as behavioral memory [26]; indicating that different intracellular signaling pathways may be involved in mediating fear and chronic pain.

In addition to assay baseline neuronal changes after the injury, some of these makers can be also used to evaluate allodynic responses in animals with the injury. In animals at two weeks after the injury, most of IEGs activities return to baseline level in the ACC. Non-noxious stimuli that usually did not induce any obvious behavioral response and gene expression in the ACC triggered remarkable IEG activation within the ACC [see [27]] (Figure 3). Similar studies in adult rats with nerve injury have reported that mild noxious stimuli triggered significant activation of phosphorylated-*c-Jun* activity in the cingulate cortex [28].

## 2. Cortical potentiation

Glutamate is the major excitatory fast transmitter in the cortical areas including the ACC [29]. Postsynaptic synaptic responses are mainly mediated by postsynaptic glutamate  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors with a small portion of currents are mediated by kainate (KA) receptors [29]. It has been proposed that excitatory synapses in the ACC undergo long-term potentiation (LTP) after peripheral injury [21,8]. Considering ACC neurons show wide spread activation to peripheral injury [22], it is much easier to detect changes in excitatory synaptic transmission in the ACC area. To explore whether there is any change in basal synaptic transmission within the ACC after nerve injury, AMPA receptor-mediated EPSCs in pyramidal neurons in the layer II/III of the ACC are measured in mice with peripheral nerve ligation [30]. Recorded neurons were identified as pyramidal neurons based on their ability to show spike frequency adaptation in response to the prolonged depolarizing-current injection [31-33]. Indeed, we found that input (stimulation intensity)-output (EPSC amplitude) curve of AMPA receptor-mediated current was significantly shifted to the left after peripheral nerve injury, compared with that in control group (Figure 4). These results suggest that excitatory synaptic transmission was increased in the ACC after peripheral nerve injury.





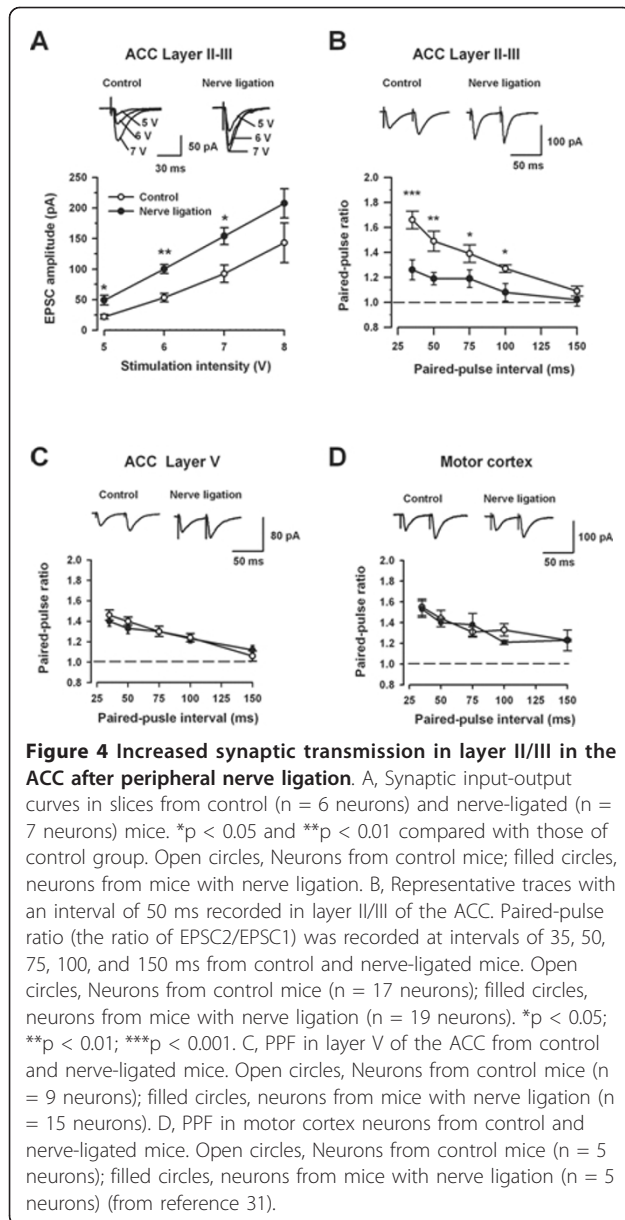
**Figure 3 Enhanced Erk activation in the ACC after tissue and nerve injury.** A. Immunohistochemical staining for phosphorylation of Erk illustrated time course-dependent activation of Erk in layer II neurons of the contralateral ACC after unilateral hindpaw injection of formalin (5%, 50 μl, n = 4-5 rats for each time point). B. The P-Erk expression in the layer II ACC neurons and their main apical dendrites (arrows) was increased at 2 weeks after the amputation of the unilateral hindpaw third digit (n = 5), compared to sham animals (n = 3). C. Mechanical stimulation by brushing hindpaw of digit amputation induced P-Erk expression in more number of layer II ACC neurons and the more distinctive apical dendrites at 2 weeks after the amputation (n = 5), compared to that in rats with amputation alone. There was not P-Erk activation in the ACC in normal animals after the brushing (n = 3). Left and middle columns: low power of the coronal ACC sections. Scale bar = 50 μm; Right column: enlarged layer II regions corresponding to the small rectangle areas in the middle column, respectively. Scale bar = 25 μm (from Reference 22).

Similar changes are found in ACC neurons in animal models of inflammation induced by complete Freund's adjuvant (CFA) [34]. CFA injection caused significant potentiation of the input-output relationship of the glutamate mediated excitatory transmission in the ACC of adult mice. Similar changes were found in animals with inflammation in rats (CFA model). Bie et al (2010) reported that CFA inflammation in rats triggered increased AMPA receptor mediated responses in ACC

neurons with observed leftward shift of the input-output curves [35].

### 3. Presynaptic enhancement of glutamate release

Paired-pulse facilitation (PPF) is a transient form of plasticity commonly used as a measure of presynaptic function, in which the response to the second stimulus is enhanced as a result of residual calcium in the presynaptic terminal after the first stimulus [34,36]. In



control mice, PPF was observed at different stimulus intervals of 35, 50, 75, 100, and 150 ms. After nerve ligation, there was a significant reduction in PPF in ACC neurons compared with those from control mice. The changes in PPF ratio is selective for neurons in layer II/III, no obvious changes were detected in deeper neurons in the ACC. These results indicate that presynaptic enhancement of the excitatory synaptic transmission selectively occurs in the layer II/III of the ACC after nerve injury (Figure 4). Similar changes in PPF ratio are found in ACC neurons of animals with CFA inflammation [33], indicating that presynaptic enhancement of glutamate release is also shared by peripheral inflammation.

In addition to the use of PPF in the ACC after peripheral nerve injury, AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) in ACC neurons in the presence of 0.5  $\mu$ M tetrodotoxin (TTX) were also found to be affected. After peripheral nerve injury, there was an obvious increase of mEPSC frequency in ACC neurons compared with that of control group. Furthermore, there was significant difference in the amplitude of mEPSCs between the two groups, indicating postsynaptic changes in AMPA receptor mediated responses (see below). By contrast, no significant change in mEPSCs amplitudes is detected in animal model of inflammation [34]. However, this does not completely rule out possible postsynaptic changes that may contribute to inflammatory pain in the ACC.

In a recent study, the use of c-Fos transgenic mice allows one to selectively record pain-trigger cortical cells [37]. The combination of c-Fos transgenic mice and PPF can help to detect selective changes in pain-activated synapses.

#### 4. Postsynaptic glutamate mediated responses

##### AMPA receptor

AMPA receptors without GluR2 are  $\text{Ca}^{2+}$  permeable and inwardly rectifying [38-40]. Inward rectification occurs by voltage-dependent blockade by polyamines [41]. To identify whether there are inwardly rectifying properties of AMPA receptors as a result of an alteration of their subunit composition in ACC neurons after nerve injury, AMPA receptor-mediated EPSCs were induced at the holding potentials of -65, -5, and +35 mV in ACC neurons. We found that there was significant difference in the rectification of AMPA receptor-mediated transmission in the ACC between control and nerve-ligated mice [36]. Consistently, when the mean current-voltage (I-V) relationship was plotted, less inward currents were found in ACC neurons from mice with nerve injury compared with control mice [36]. These results demonstrate that AMPA receptor in ACC neurons has an inward rectification property in neuropathic pain.

Similar rectification of the AMPA receptor mediated responses in ACC neurons of rats has been reported after peripheral inflammation with hindpaw CFA injection [35].

##### Postsynaptic NMDA NR2B receptor

Evidence for the involvement of cortical NMDA NR2B receptor in chronic pain first comes from the NMDA NR2B forebrain overexpressed 'smart' mice [42,43]. In this transgenic mouse with selective forebrain NMDA NR2B overexpression, inflammatory pain and allodynia were significantly enhanced without any significant effect on acute pain. Subsequent studies demonstrated that peripheral inflammation with CFA injection triggered long-lasting increases in the expression of NMDA NR2B receptor proteins in the ACC [44,45]. The

changes in NMDA receptor protein expression are subtype selective, since other NMDA receptor subunits such as NR1 and NR2A did not show any significant change (Figure 5). The increased NR2B receptors are likely to be within synapses, because single-shock focal stimulation induced NR2B receptor-mediated synaptic currents were enhanced. Furthermore, a recent study reported that gentiopicroside, an analgesic compound for inflammatory pain, reduced the expression of ACC NR2B induced by the injury, as well as NMDA receptor mediated responses [45].

In an animal model of arthritic pain, it has been reported that inflammation increased phosphorylation but not up-regulation of NMDA NR1 proteins in the central amygdala [46]. In parallel, NMDA receptor mediated responses are also enhanced.

#### **Sensitivity to inhibition by protein kinase M zeta (PKM $\zeta$ ) inhibitor ZIP**

What is likely to be the synaptic mechanism responsible for the analgesic effects produced by ZIP in neuropathic pain? PKM $\zeta$  can potentiate postsynaptically the amplitude of AMPA receptor-mediated EPSCs [37,47]. Because glutamatergic synaptic transmission in the ACC is increased after nerve injury [36], we speculated that PKM $\zeta$  may contribute to the maintenance of enhanced synaptic transmission induced by nerve injury. First, we recorded AMPA receptor-mediated EPSCs in layer II/III of the ACC 3 or 7 days after nerve injury [37]. We found that bath application of ZIP (5  $\mu$ M) significantly reduced eEPSCs (Figure 6). In contrast, ZIP did not affect the amplitude of eEPSCs recorded in neurons from sham-operated mice.

## **5. Biochemical markers**

### **Membrane GluR1 expression**

The trafficking of AMPA receptor subunits has been proposed to contribute to synaptic plasticity underlying hyperalgesia [48-50]. We investigated the distribution of AMPA receptor subunits in the ACC after nerve ligation. We found that induction of neuropathic pain by nerve ligation was associated with an increase in the abundance of the GluR1 subunits in the membrane fraction and a corresponding decrease in the levels in the cytosolic fraction. In contrast, nerve ligation had no effect on the intracellular distribution of GluR2/3 subunits in ACC neurons [36]. The data show that AMPA receptor GluR1 subunit is redistributed in ACC neurons as a result of nerve injury.

In rats with CFA inflammation, Bie et al reported that AMPA GluR1 in the synapsome preparation of the ACC neurons from rats with CFA injection showed significant increases, suggesting that synaptic AMPA GluR1 is significantly increased [35].

### **Phosphorylation of GluR1**

The phosphorylation of GluR1 subunit of AMPA receptors is critical for synaptic expression of the receptors, their channel properties, and synaptic plasticity [51-53]. We tested the phosphorylation levels of GluR1 subunit at the PKA phosphorylation site (Ser 845) in the ACC of the mice with nerve ligation. We found that the phosphorylation levels of GluR1 were significantly increased in the ACC after nerve injury. The data indicate that the nerve injury can increase the phosphorylation levels of GluR1 through the PKA signaling pathway.

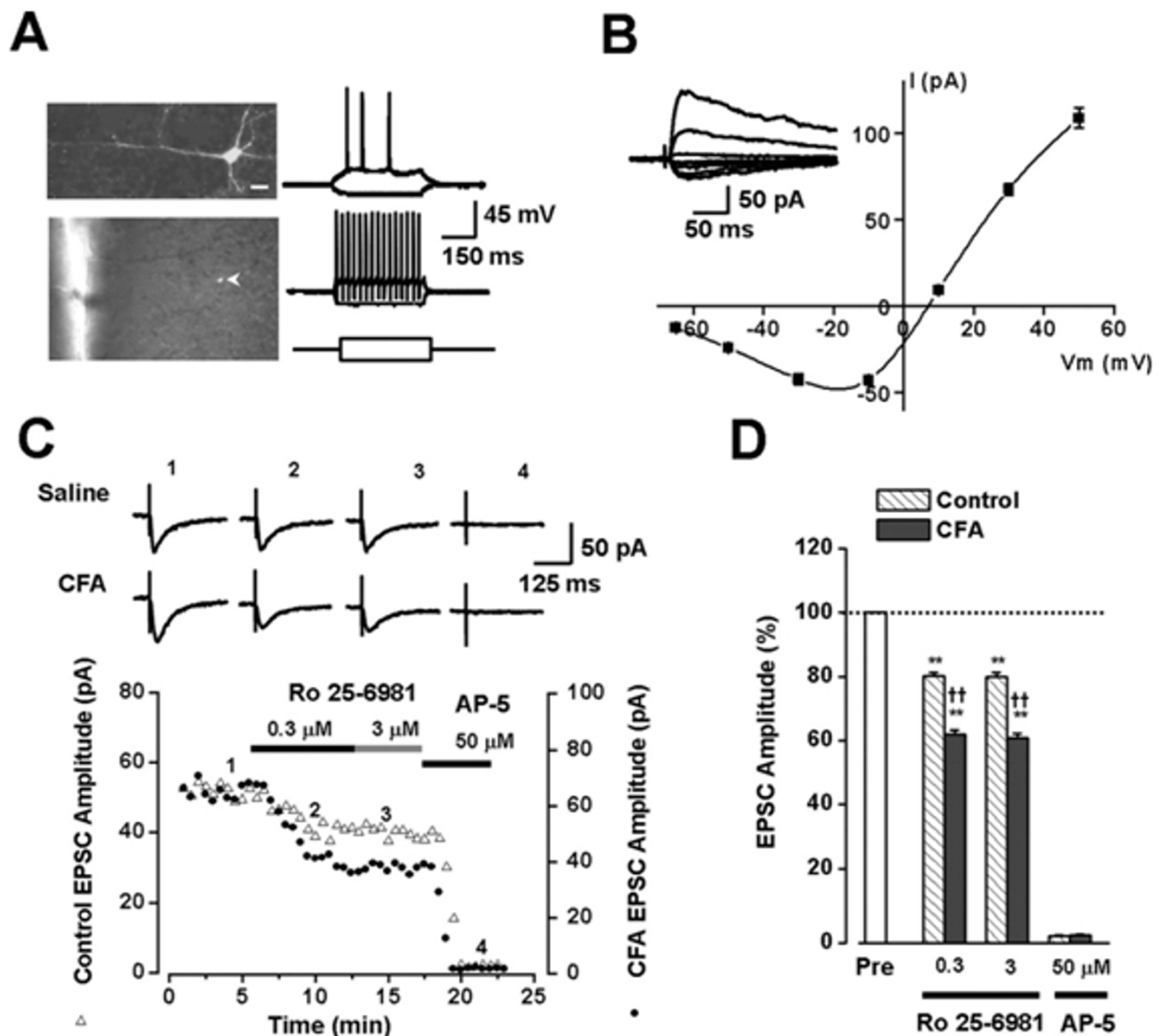
### **Upregulation of PKM $\zeta$ as an marker**

We examined whether, in the ACC, peripheral nerve injury causes changes in PKM $\zeta$  [37]. Behavioral allodynic response was increased 3 days after nerve injury when compared with the response in sham-treated mice. The levels of PKM $\zeta$  in the ACC were significantly increased after nerve injury. Because PKM $\zeta$  is activated by phosphorylation, we also conducted experiments to detect possible changes in the level of phosphorylated PKM $\zeta$  (p-PKM $\zeta$ ). Consistently, the level of p-PKM $\zeta$  was also significantly increased. These data suggest that peripheral nerve injury increases PKM $\zeta$  activity in the ACC. To determine whether such changes are long-lasting, we examined PKM $\zeta$  and p-PKM $\zeta$  levels 7 and 14 days after nerve injury. Although behavioral allodynia persisted at these time points, the protein levels of PKM $\zeta$  returned to baseline (Figure 7), indicating that the regulation of the amount of PKM $\zeta$  is short-lasting. However, the p-PKM $\zeta$  level remained increased, suggesting that PKM $\zeta$  activity could contribute to the maintenance of neuropathic pain. To investigate whether the changes in PKM $\zeta$  protein levels or activity were a generalized phenomenon in the central nervous system, we also examined the levels of PKM $\zeta$  and p-PKM $\zeta$  in the hippocampus and spinal dorsal horn 3 days after nerve injury.

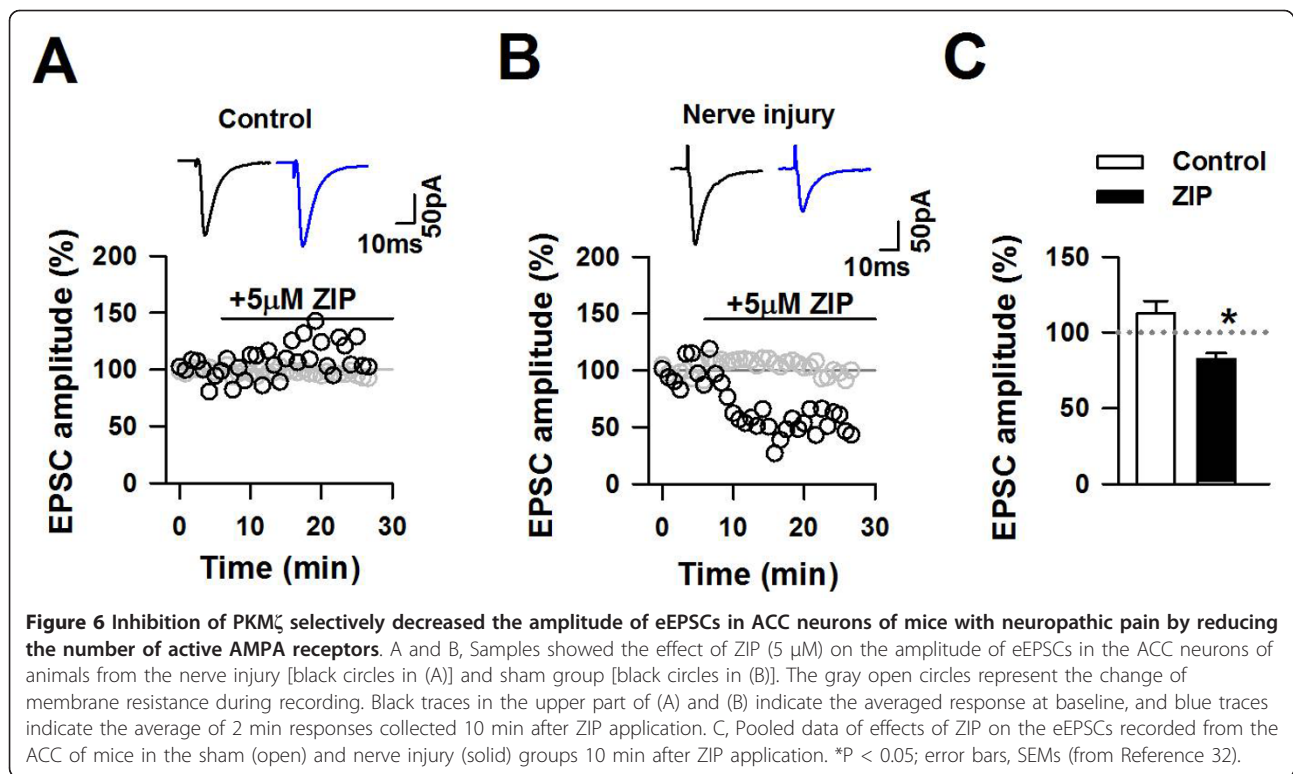
## **6. In vivo electrophysiological as direct evidence for chronic pain**

In brain slices, cingulate synapses can undergo LTP after experimentally designed training protocols. One key question regarding ACC plasticity is whether or not injury causes long-term changes in synaptic transmission in the ACC in intact animals. To test this, we performed experiments in anesthetized rats. We measured synaptic responses to peripheral electrical shocks by placing a recording electrode in the ACC of anesthetized rats [54]. At high intensities of stimulation, sufficient to activate nociceptive A $\delta$  and C fibers, evoked field EPSPs were recorded in the ACC. Digit amputation at the contralateral hindpaw causes a rapid and long-lasting enhancement (more than 120 min) of sensory responses (Figure 8). Potentiated sensory responses do not require





**Figure 5 Increased NMDA NR2B receptor-mediated postsynaptic currents in adult ACC neurons is shown after inflammation.** A, Confocal image of a pyramidal neuron in an adult ACC slice loaded with Lucifer yellow (top left). The arrow indicates the location of the neuron in the ACC slice (bottom left). Scale bar, 20  $\mu$ m. When injected with current steps from -100 to 100 pA in 400 ms (bottom right), the neuron shown at the left fired repetitive action potentials with frequency adaptation (top right). A minority of neurons recorded ( $n = 4$ ) showed the fast-spike properties (middle right). B, Current-voltage plot of the NMDA receptor EPSCs. The currents exhibited strong outward rectification and the reversal potential of the linear part was  $4.4 \pm 2.0$  mV ( $n = 6$  neurons/5 mice). Inset, NMDA receptor-mediated EPSCs recorded at holding potentials from -65 to 50 mV. Each trace represents the average of five consecutive recordings. C, A selective NR2B antagonist, Ro 25-6981, partially inhibited NMDA receptor-mediated EPSCs. The time course of changes in EPSC amplitude before and during the application of Ro 25-6981 (0.3 and 3  $\mu$ M) and AP-5 (50  $\mu$ M) in ACC neurons from both saline (control,  $\Delta$ ) and CFA-injected ( $\square$ ) mice is shown. Traces show the currents at different time points during application of drugs. Ro 25-6981 produced its maximal effect at 3 min after bath application, and a higher dose of Ro 25-6981 (3  $\mu$ M) had no additional effects. The remaining currents can be totally blocked by AP-5 (50  $\mu$ M). D, Summary data of the effects of Ro 25-6981 (0.3 and 3  $\mu$ M) in ACC neurons of control (saline-injected) and CFA-injected mice. Ro 25-6981 produced significantly greater inhibitory effects on NMDA receptor-mediated EPSCs in ACC neurons in CFA-injected mice ( $n = 9$  neurons/8 mice) than those in control adult mice ( $n = 8$  neurons/7 mice). AP-5 (50  $\mu$ M) completely blocked the currents, confirming the currents are mediated NMDA receptors ( $n = 5$  slices/5 mice). The double asterisks indicate significant difference from control, and the double daggers indicate significant difference from control NMDA receptor-mediated EPSCs. Error bars represent SEM (from Reference 24).



persistent activity from the injured hindpaw [54]. These findings indicate that plastic changes are likely occurring within the ACC synapses. Furthermore, *in vivo* intracellular recordings from anesthetized rats have confirmed similar findings [55].

### 7. Long-term structural changes

It has been known that learning related plasticity requires transcriptional and translational processing and triggers long-term structural changes in individual synapses [56]. Considering wide-spreading neuronal plasticity happens in the central nervous system after a peripheral injury, it is expected to find long-term changes in brain areas related to chronic pain. The well known cortical reorganization of somatosensory cortex was reported in monkeys with amputated arms. Recently, in patients with chronic pain such as chronic back pain, loss of cortical areas has been reported [7,57].

In animal models of inflammatory pain or chronic pain, it has been reported that inhibiting macromolecular synthesis is analgesic [58]. Structural or synaptic changes after nerve injury have also been reported in cortical areas [59,60]. Metz et al reported that layer 2/3 pyramidal neurons in acute slices of the contralateral medial prefrontal cortex (mPFC) in the rat spared nerve injury model of neuropathic pain showed morphological differences between the mPFC of injured and sham-operated animals [60]. Basal dendrites of neurons from injured rats are

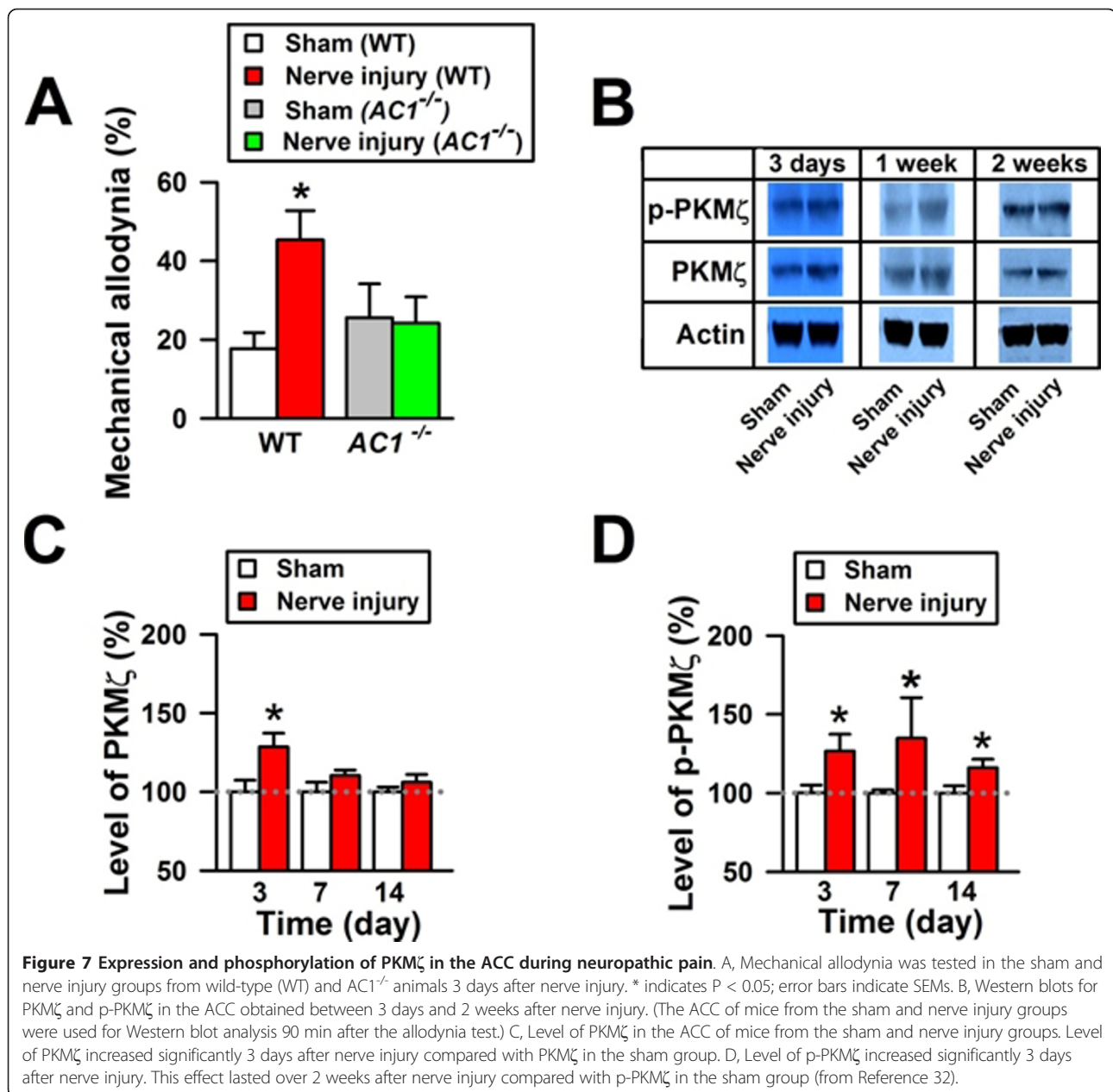
longer and have more branches than their counterparts in sham-operated animals; spine density is also selectively increased in basal dendrites of neurons from injured rats.

### 8. Imaging ACC activity and spike recording in experimental animals

Electrophysiological recordings of sensory induced unit responses from cortical neurons is another direct measure of injury induced cortical changes. The use of responsive threshold, the measurement of receptive field, and changes in the magnitude of nociceptive responses are good indexes for injury related cortical plasticity. It is likely that heterogeneous populations of cells are likely found. For example, in the amygdala, Neugebauer and Li reported (2002) that amygdala neurons showed differential sensitization (measured by spike activities) to sensory afferent inputs in a model of arthritic pain [61].

In addition to spike recordings, recent studies using whole-cell patch-clamp recordings from adult ACC neurons found that many of ACC neurons are responsive to peripheral nociceptive stimulation. Whole-cell patch-clamp recording offer better sensitivity for detecting possible changes in chronic pain, and evaluate the possible analgesic effects of drugs [62].

Recent several studies have reported brain activation in awake rats using the imaging technique [63]. In this novel approach, Becerra et al (2010) performed fMRI in trained, acclimated, awake rats [63]. The new approach

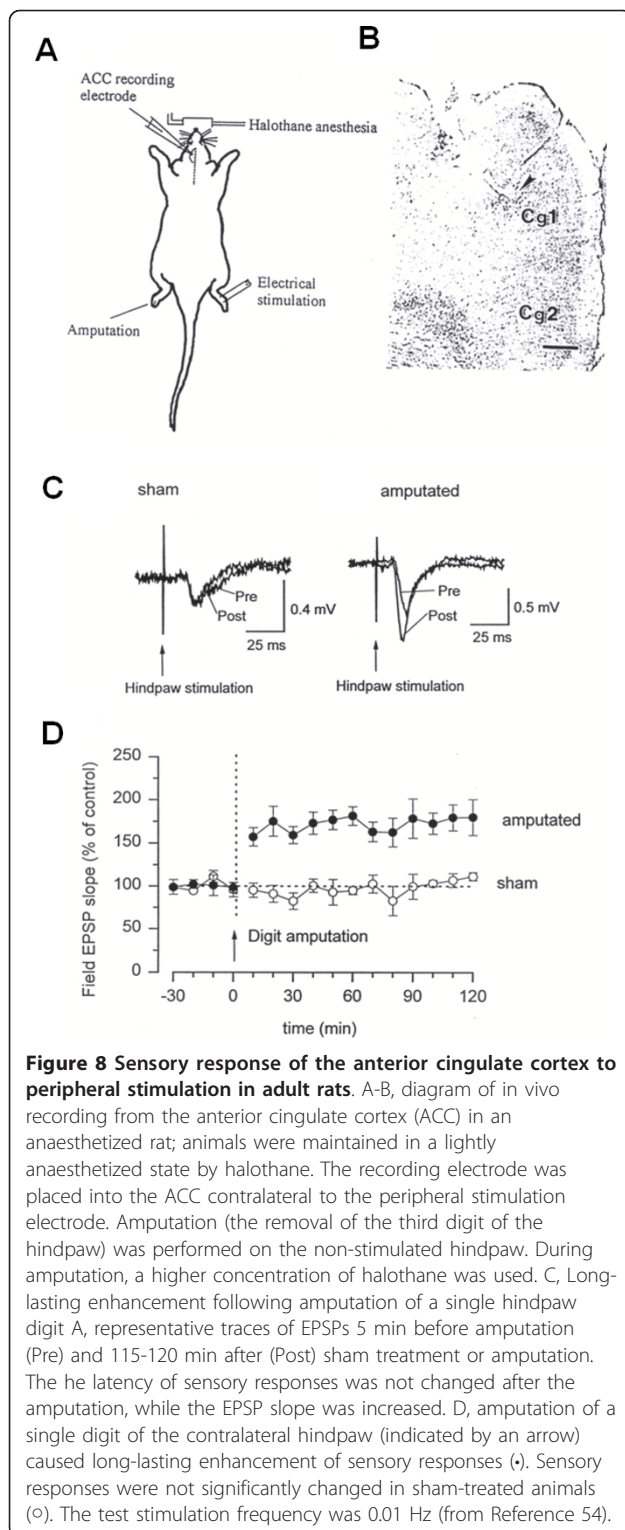


avoids the potential complicating effects of anesthesia. Differing from experiments in humans, animal needs to be kept under anesthesia state to avoid the movement. Among many cortical areas, they reported that ACC, somatosensory cortex and IC are activated. This approach can be effectively used for evaluating effects of new drugs on pain in awake animals.

#### Examples how cortical endpoints can be used

It is known that injury triggers molecular and cellular changes in different parts of the brains. Depending on the specific regions of the brain, these neurobiological changes may contribute to different aspects of pain,

such as learning and memory, anxiety, unpleasantness and attention. Although some of these changes can be observed at behavioral levels (i.e., reduced latency to withdrawal; or enhanced responses to noxious stimuli), some are difficult to be studied at behavioral level. One good example to use the cortical endpoints is to evaluate the effects of new drugs in spinal cord injury induced pain. It is commonly reported in patients with spinal cord injury that they suffer long-lasting neuropathic pain caused by the injury. However, in animal models of spinal cord injury, it is impossible to evaluate the drug's analgesic effect using behavioral tests. Using the proposed cortical endpoints, I propose that these



cortical endpoints can be used. For example, activation of various IEGs in the pain-related cortex such as ACC can be used to evaluate early activation, and measuring glutamate mediated responses and biochemical markers can be used to evaluate enhanced transmission during

chronic pain induced by spinal cord injury. The drugs that inhibited or erased cortical markers may be potentially useful for treating spinal cord injury related chronic pain in patients.

The same endpoints can be used to evaluate drugs that acting at peripheral or spinal mechanisms. In additional behavioral responses, cortical endpoints can be measured before and after drug application. Drugs that reduce peripheral or spinal sensitization or potentiation should reduce activation of cortical markers. The cortical measurement will help to determine if behavioral inhibition is due to pure inhibition of motor neurons that are required for sensitized withdrawal responses in chronic pain conditions. There are some limitations of using these proposed cortical indexes to study 'pain' in animals. It is important to perform additional experiments to distinguish possible changes in these cortical indexes that are important for pain-related memory but not pain itself. Considering that cortical neurons are often activated in different situations, it is not an easy task if the injury is also located within the brain. The use of selective gene-manipulated mice or pharmacological inhibitors may help to address some of these concerns.

## Conclusion

In summary, recent basic neurobiological investigations of physiological and pathological mechanisms of pain and chronic pain provide critical information for our understanding of long-term plastic changes in cortical areas. These cortical changes that persisted during the course of chronic pain can be used a valuable index to measure 'pain' in animals. The combined use of these neurobiological indexes, together with or without behavioral motor withdrawal responses, will greatly facilitate our searching for new drugs, and also help us to understand why some of current pain medicine does not work in some clinical conditions.

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## Authors' contributions

MZ designed and finished the manuscript.

## Competing interests

The author declares that they have no competing interests.

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