Loss of long-term depression in the insular cortex after tail amputation in adult mice

Ming-Gang Liu1,2 and Min Zhuo1,2*

Abstract
The insular cortex (IC) is an important forebrain structure involved in pain perception and taste memory formation. Using a 64-channel multi-electrode array system, we recently identified and characterized two major forms of synaptic plasticity in the adult mouse IC: long-term potentiation (LTP) and long-term depression (LTD). In this study, we investigate injury-related metaplastic changes in insular synaptic plasticity after distal tail amputation. We found that tail amputation in adult mice produced a selective loss of low frequency stimulation-induced LTD in the IC, without affecting (RS)-3,5-dihydroxyphenylglycine (DHPG)-evoked LTD. The impaired insular LTD could be pharmacologically rescued by priming the IC slices with a lower dose of DHPG application, a form of metaplasticity which involves activation of protein kinase C but not protein kinase A or calcium/calmodulin-dependent protein kinase II. These findings provide important insights into the synaptic mechanisms of cortical changes after peripheral amputation and suggest that restoration of insular LTD may represent a novel therapeutic strategy against the synaptic dysfunctions underlying the pathophysiology of phantom pain.

Background
Insular cortex (IC) is an integrating forebrain structure involved in several sensory and cognitive functions, such as interoceptive awareness, taste memory, and pain perception [1-3]. In particular, human brain imaging studies have demonstrated the activation of IC in a broad range of pain conditions [4-6]. Moreover, electrical stimulation of IC directly elicits painful sensations in human subjects [7-9]. The involvement of IC in chronic pain has also been confirmed by animal experiments, showing the presence of nociceptive neurons [10,11] and pain-evoked biochemical changes [12,13] in this area. Genetic [14,15] or pharmacological [16-19] manipulation of the IC could alter the pain sensitivity. Importantly, long-term potentiation (LTP) has been revealed in the IC by both in vivo [20,21] and in vitro [22,23] electrophysiological recordings. Furthermore, neuropathic pain experience could occlude the electrical induction of insular LTP in adult mice [18], suggesting that chronic pain may share common mechanisms with insular synaptic plasticity [24].

Phantom pain refers to the feeling of pain in a body part that has been amputated [25-27]. Mechanistically, limb amputation has been shown to cause dramatic cortical reorganization in humans and primates [28-31], the amount of which correlates well with the extent of phantom pain in some reports [32-34]. We previously demonstrated that digit amputation in rats or tail amputation in mice triggered long-lasting plastic alterations in the anterior cingulate cortex (ACC), including an enhancement of excitatory synaptic responses in vivo [35,36], loss of long-term depression (LTD) in vitro [37,38] and activation of activity-dependent immediate early genes [37,39]. In addition to ACC, human imaging studies also revealed a correlation between the IC activation and phantom pain [25,40,41]. Thus, it is important to investigate the possible changes in synaptic plasticity in the IC after amputation.

It is believed that peripheral injury elicits long-lasting plastic changes in the brain via at least two major mechanisms: one is direct enhancement of excitatory synaptic transmission, and the other is loss of the ability to undergo LTD [42, also see Table 1]. In the present study, we used a 64-channel multi-electrode dish (MED64) recording system [23,38,43] to examine injury-related metaplastic changes in insular LTD caused by tail amputation in the adult mice. Our previous results demonstrated the coexistence of two different forms of LTD in the IC:
NMDA receptor-dependent LTD and NMDA receptor-independent LTD [44]. Here, we report that tail amputation produces a selective loss of low frequency stimulation (LFS)-induced LTD in the adult mice IC, leaving (RS)-3,5-dihydroxyphenylglycine (DHPG)-evoked LTD intact. The impaired insular LTD could be pharmacologically rescued by priming the IC slices with application of a low-dose group I metabotropic glutamate receptor (mGluR) agonist DHPG, a form of metaplasticity that involves activation of protein kinase C (PKC) but not protein kinase A (PKA) or calcium/calmodulin-dependent protein kinase II (CaMKII).

**Results**

**Loss of LFS-evoked LTD in the IC after tail amputation**

Our previous work has demonstrated that digit or tail amputation in rats or mice could abolish the induction of LFS-evoked LTD in the ACC [37,38]. Here, we employed a previously-established 64-channel multi-electrode array system, i.e. the MED64 system [23,38,44], to examine whether the insular synaptic plasticity is equally sensitive to the amputation-induced peripheral injury in adult mice. MED64 recordings were performed in the IC slices obtained from sham control or tail-amputated mice at 2 weeks after surgery (Figure 1A). The relative location of the MED64 probe within the IC slice is shown in Figure 1B. We focused our recording sites on the rostral IC region at the level of the corpus callosum connection, where the stimulation site is usually located in the deep layer (layer V-VI) of the IC slice (the red dot in Figure 1B). One representative example recording is illustrated in Figure 1C (before LFS) and Figure 1D (60 min after LFS) for the tail-amputated group. It is clearly discerned that LFS failed to induce any depression of field excitatory postsynaptic potential (fEPSP) in this slice. The averaged data showed a complete loss of LFS-evoked LTD in the superficial layer (96.8 ± 1.9% of baseline at 60 min after LFS, n = 9 slices/5 mice, P = 0.701, Student's t-test, Figure 1E). In contrast, the sham control group exhibited clear LTD of multisite synaptic responses (66.2 ± 2.5% of baseline, n = 9 slices/7 mice, P < 0.001, Student's t-test, Figure 1E), which is consistent with our previous publication [44].

Neurons in different layers of the IC are considered to have different afferent and efferent connections with other areas of the brain, and thus may mediate distinct functions [48-50]. Therefore, we next asked whether tail amputation could also affect the LTD induction in the deep layer as it did in the superficial layer. As previously described [44], LFS application produced a long-lasting synaptic depression of fEPSPs recorded in the deep layer of the IC from the sham control group (70.3 ± 1.5% of baseline at 60 min after LFS, n = 9 slices/7 mice, P < 0.001, Student's t-test, Figure 2A). However, LFS failed to induce any LTD in the tail-amputated group (94.0 ± 2.6% of baseline, n = 9 slices/5 mice, P = 0.137, Student's t-test, Figure 2A). Statistical analysis revealed a strong significant difference between sham and tail-amputated groups in the degree of LTD in both superficial layer (P < 0.001, Student's t-test, Figure 1E) and deep layer (P < 0.001, Student's t-test, Figure 2A).

Furthermore, we analyzed the induction ratio of insular LTD, defined as the percentage of LTD-showing channels among all activated channels. This ratio was greatly decreased in the tail-amputated slices (superficial layer: 15.5 ± 2.9%, P < 0.001, Student's t-test; deep layer: 17.7 ± 4.8%, P < 0.001, Student's t-test) compared to the sham control (superficial layer: 59.4 ± 6.0%; deep layer: 48.9 ± 4.7%, Figure 2B). Similar results were obtained with stimulation at another frequency (5 Hz, 3 min; data not shown). These findings suggest that tail amputation results in an inability of insular synapses to undergo LTD, regardless of the specific layer.
Lack of the effect of amputation on DHPG-induced insular LTD

Recently, we reported the co-existence of two distinct forms of LTD in the insular synapses: NMDA receptor-dependent LTD induced by LFS, and NMDA receptor-independent LTD induced by DHPG application [44]. Next, we sought to examine whether tail amputation could also affect the induction of DHPG-LTD. We induced DHPG-LTD by bath application of 100 μM DHPG for 20 min and then washed it out to monitor the course of chemically-induced LTD for 50 min. Similar to the previous study, DHPG infusion produced a rapid and long-lasting depression of fEPSP in the IC slices (Figure 3A and B). The synaptic responses of the superficial layer were reduced to 72.5 ± 1.8% of baseline (n = 7 slices/7 mice, P < 0.001, Student’s t-test, Figure 3A) at 50 min after washout of DHPG in the sham group. Interestingly, we did not observe any abolition of DHPG-LTD in the IC after tail amputation (73.6 ± 2.1% of baseline, n = 9 slices/9 mice, P < 0.001, Student’s t-test, Figure 3A). Similarly, the lack of effect of amputation on DHPG-LTD is also replicated in the deep layer of the IC (sham control: 77.1 ± 3.0% of baseline, n = 8 slices/8 mice, P = 0.303, Student’s t-test, Figure 3B). The magnitude and duration of DHPG-LTD in the tail-amputated group did not differ from the sham control (superficial layer: P = 0.311, Student’s t-test, Figure 3A; deep layer, P = 0.303, Student’s t-test, Figure 3B). Likewise, the induction ratio
of DHPG-LTD in the IC was not different between the two groups in either superficial layer (sham vs. tail amputation: 49.2 ± 10.1% vs. 47.6 ± 6.7%, P = 0.431, Student’s t-test) or deep layer (sham vs. tail amputation: 63.6 ± 8.1% vs. 59.6 ± 6.9%, P = 0.711, Student’s t-test, Figure 3C). Taken together, these results suggest that tail amputation selectively blocked the induction of LFS-evoked insular LTD, with the DHPG-LTD being intact. This result in the IC is in contrast to that in the ACC, where tail amputation prevented the occurrence of both LFS-induced LTD and mGluR1-mediated LTD [38].
Enhanced synaptic transmission in the IC after tail amputation

It has been previously reported that peripheral inflammation or nerve injury could trigger a long-term enhancement of excitatory synaptic transmission in various brain regions, such as ACC [51-54], amygdala [55-57], and hippocampus [58]. We next examined whether similar alterations in synaptic efficacy could be elicited in the IC after peripheral injury. The input–output relationships, measuring fEPSP slope (output) as a function of the afferent stimulus intensity (input), were compared between sham control and tail-amputated (two weeks) groups. The slope of the curve was evidently shifted to the left at higher stimulation intensities after amputation (n = 6 slices/4 mice for both superficial layer and deep layer), compared with that in control group (n = 6 slices/6 mice for superficial layer; n = 5 slices/5 mice for deep layer) (Figure 4A and B). These results suggest that excitatory synaptic transmission is likely enhanced following tail amputation experience. Nevertheless, the curves did not move leftward in a parallel manner, indicating no alteration in the threshold for inducing fEPSPs. Furthermore, the input–output curves of the total number of activated channels showed a similar trend between the two groups (n = 6 slices/6 mice for sham control; n = 8 slices/5 mice for tail-amputated group; Figure 4C).

Pharmacological rescue of LFS-evoked insular LTD after tail amputation

Prior activation of group I mGluRs could produce metaplastic effects on synaptic plasticity in the hippocampus, shown as a large enhancement in the induction of hippocampal LTP [59,60, for review, see 61]. Our previous work revealed another form of group I mGluR-mediated metaplasticity in the ACC, that is, priming ACC slices with pharmacological activation of mGluR1 rescued the loss of LTD caused by the tail amputation [38]. Here, using the same rationale, we attempted to rescue LFS-induced insular LTD by priming the IC slices with bath application of a lower dose of DHPG (20 μM, 20 min). Figure 5A and B illustrates the overview of the 64-channel recordings obtained before LFS and 60 min after LFS in one DHPG-primed and tail-amputated IC slice. DHPG treatment at this dose failed to trigger any LTD of multisite synaptic responses, but only a rapid and transient acute depression was observed in either superficial layer (90.8% of baseline at the end of DHPG infusion, Figure 5C) or deep layer (92.4% of baseline at the end of DHPG infusion, Figure 5D). However, subsequent LFS indeed led to a significant depression of the fEPSPs in a single example (superficial layer: 70.0% of baseline at 60 min after LFS, Figure 5C; deep layer: 65.9% of baseline at 60 min after LFS, Figure 5D) and in pooled data (superficial layer: 67.5 ± 3.5% of baseline, n = 6 slices/5 mice, P = 0.002, Student’s t-test, Figure 5E; deep layer: 67.5 ± 2.9%...
of baseline, n = 5 slices/5 mice, P = 0.008, Student’s t-test, Figure 5F). The magnitude of DHPG-rescued LTD in the tail-amputated mice is similar to that of the sham control mice (compare Figure 5E and F with Figure 1E and Figure 2A). These results indicate that similar to the ACC synapses, prior activation of group I mGluRs can produce a form of metaplasticity that restores the LFS-evoked LTD in the IC in the tail-amputated mice.

**Protein kinase C, but not CaMKII or PKA, is involved in the rescue of insular LTD**

To probe the mechanisms underlying the metaplastic rescue of LFS-evoked LTD in the IC, we next performed pharmacological experiments using different protein kinase inhibitors, based on previous reports showing the critical roles of various protein kinases in mediating multiple forms of metaplasticity in the hippocampus [for reviews, see 61,62]. At first, we examined the involvement of PKC in the DHPG-induced priming effect, given the increasing evidence supporting the role of PKC in metaplasticity [63-65]. Co-application of a PKC inhibitor chelerythrine (Che, 3 μM) with the DHPG (20 μM, 20 min) prevented the rescue of LTD in both superficial layer (96.2 ± 2.4% of baseline within the last 10 min of recording, n = 6 slices/6 mice, P = 0.006, One-Way ANOVA followed by Fisher’s LSD test, Figure 6B and E) and deep layer (96.0 ± 1.4% of baseline within the last 10 min of recording, n = 5 slices/3 mice, P < 0.001, One-Way ANOVA followed by Fisher’s LSD test, Figure 7B and E) of the IC slice taken from tail-amputated mice. In contrast, simultaneous treatment of the IC slice with vehicle had no effect on the LTD rescue (superficial layer: 71.0 ± 2.9% of baseline, n = 5 slices/3 mice, Figure 6A and E; deep layer: 79.3 ± 1.9% of baseline, n = 5 slices/3 mice, Figure 7A and E).

Besides PKC, CaMKII and PKA have also been shown to mediate certain forms of metaplasticity [66-68]. Therefore, we also evaluated the role of these two kinases in
DHPG-rescued insular LTD in the tail-amputated mice. As shown in Figure 6C-E, neither KN62 (5 μM, a CaMKII inhibitor) nor KT5720 (1 μM, a PKA inhibitor) could block the induction of LTD in the superficial layer of the IC (KN62: 65.0 ± 3.5% of baseline, n = 6 slices/5 mice, P = 0.595, One-Way ANOVA followed by Fisher’s LSD test; KT5720: 72.6 ± 3.9% of baseline, n = 7 slices/5 mice, P = 0.558, One-Way ANOVA followed by Fisher’s LSD test). Similar results were obtained in the deep layer (KN62: 72.8 ± 2.6% of baseline, n = 6 slices/5 mice, P = 0.382, One-Way ANOVA followed by Fisher’s LSD test; KT5720: 81.9 ± 1.9% of baseline, n = 7 slices/5 mice, P = 0.544, One-Way ANOVA followed by Fisher’s LSD test, Figure 7C-E). These observations are consistent with our previous results in the ACC [38], suggesting that PKC, but not CaMKII or PKA, acts as a major mediator in mGluR-evoked metaplasticity in the IC in tail-amputated animals.

**Discussion**

There is considerable evidence indicating the critical role of the IC in pain perception and memory storage [2,3,16-18]. However, few studies have been conducted at the cellular level to address the synaptic basis of IC-mediated higher brain functions. Our recent work demonstrates that fast excitatory synaptic transmission in the IC is mainly mediated by postsynaptic AMPA/kainate receptors and that both LTP and LTD could be induced reliably but with different receptor mechanisms [23,44,48]. Since cortical plasticity has been proposed to be an endpoint measurement and...
working mechanism of chronic pain [24,69], it would be interesting to address the metaplastic effects of chronic pain experience in vivo on the induction of insular LTP and LTD in vitro. We recently report that nerve injury-induced neuropathic pain could fully occlude the subsequent induction of LTP in the IC [18]. In the present study, using a 64-channel multi-electrode array system, we further evaluated the effect of abnormal pain processing on insular LTD. We found that a two-week experience of amputation-induced peripheral injury resulted in a selective impairment of insular LTD induction by the LFS protocol, but without any effect on DHPG-induced LTD. Priming the IC slices with pharmacological activation of group I mGluRs rescued the LFS-induced LTD after amputation, which involves the activation of PKC, but not PKA or CaMKII.

**Loss of LTD in the IC after amputation**

One of the central findings in this study is the loss of LFS-evoked LTD in tail-amputated IC slices. We selected two weeks after amputation as the time point for taking the IC slices for multi-channel recordings, mainly based on our previous publications showing the occurrence of marked plastic changes in the ACC at this time. Specifically, we found that peripheral amputation abolished LTD and enhanced extracellular signal-regulated kinase activation in the rodent ACC at two weeks [37-39]. Nevertheless, amputation-caused plastic changes in the brain might be

---

**Figure 7** PKC, but not CaMKII or PKA, is involved in the rescue of LFS-evoked insular LTD in the deep layer. (A) Vehicle control group exhibited the normal rescue of insular LTD (n = 5 slices/3 mice). (B) Co-application of the PKC inhibitor chelerythrine (Che, 3 μM) together with DHPG (20 μM, 20 min) blocked the LTD rescue (n = 6 slices/6 mice). (C) CaMKII inhibitor KN62 (5 μM) could not affect the LTD recovery (n = 6 slices/5 mice). (D) PKA inhibitor KT5720 (1 μM) had no effect on DHPG-primed insular LTD (n = 7 slices/5 mice). Inset traces in (A-D) show representative fEPSPs at the time points indicated by the numbers in the graph. Calibration: 100 μV, 10 ms. Horizontal bars denote the period of DHPG application or LFS delivery as indicated. (E) Bar histogram summarizing the averaged data within the last 10 min of the LTD recording. ***P < 0.001. NS, no significance. Error bars represent SEM.
time-dependent. For example, digit amputation can abolish ACC LTD and enhance hippocampal LTP at 45 min but failed to elicit any significant change in the hippocampus at 20 min or earlier [37,45]. Thus, future studies are clearly needed to investigate if tail amputation-induced loss of insular LTD is time dependent, and if so, when the metaplastic alterations are initiated and how long they can last.

The detailed mechanisms underlying this LTD abolition are not well understood. However, our previous work revealed a similar deficit of LTD induction in the ACC from adult rats or mice subjecting to digit or tail amputation, respectively [37,38]. In addition, tissue amputation produced a rapid and prolonged enhancement of sensory responses to noxious stimulation, dramatic membrane depolarization, as well as large-scale expression of several immediate early genes and signaling molecules in the ACC [35,37,39,70, for review, see 42]. These observations allow us to speculate that enhanced postsynaptic excitability might also occur in the IC after tail amputation, which leads to the failure of LTD induction. Supporting this assertion, we found a leftward shift of input–output curves of fEPSPs in tail-amputated slices as compared to the control group. Furthermore, our recent work demonstrates that induction of insular LTD by LFS involves activation of the NMDA receptor and mGluR5 [44]. Since DHPG-induced LTD is not affected by amputation (see below), an alternative explanation for the loss of LFS-evoked LTD might be due to the changes in the expression and/or function of NMDA receptor in the IC caused by tail amputation. Injury-induced deficits in signaling cascades at the downstream of the NMDA receptor activation may also contribute to the loss of insular LTD. Regardless of the mechanisms, loss of the ability to undergo LTD in the IC might be an essential synaptic mechanism accounting for the maladaptive central plasticity occurring after amputation [25,26,42].

**DHPG-induced LTD is not affected by tail amputation**

One unexpected finding of this study is that tail amputation did not affect the induction of DHPG-LTD in superficial and deep layers of the IC. These results stand in contrast with those obtained from the adult mice ACC slices, where both electrically-induced LTD and chemically-induced LTD were significantly impaired by tail amputation [38]. The exact reasons for these discrepancies are not clear but might be due to the differences in the mGluR-targeting drugs used (DHPG vs. DHPG + MPEP) and the forebrain regions analyzed (IC vs. ACC). The conflicting observations between LFS-and DHPG-induced insular LTD could arise from their differences in the vulnerability to amputation-elicited plastic changes in the IC area. This discrepancy is also in accordance with our recent publication, demonstrating that DHPG-LTD and LFS-induced LTD represent two distinct forms of LTD co-existing in the insular synapses and do not occlude each other [44].

It is noteworthy that region-related differences might exist when considering the effects of tissue amputation on synaptic plasticity in the pain-related brain regions (summarized in Table 1). Specifically, although either tail or digit amputation triggered a complete loss of LTD in the ACC [37,38] or the IC (the present study), almost the same manipulation has no effect on LTD induction in the hippocampus or parietal cortex [37,45]. Also, partial ligation of the sciatic nerve, a well-established animal model of neuropathic pain, does not affect the induction of LFS-evoked LTD in the hippocampus [46]. These findings indicate that both ACC and IC play important roles in amputation-related cortical plasticity, and such changes are relatively selective for pain-related areas. It is unlikely due to the general stress or other non-selective factors caused by amputation. Targeting these alterations in synaptic plasticity in the brain might provide an alternative approach for the treatment of chronic pain including the phantom pain [24,25,42,69].

**mGluR-dependent rescue of insular LTD after tail amputation**

It is now well-known that mGluRs activation is not only directly involved in the induction of LTP or LTD, but is also engaged in a process called metaplasticity, by which prior neuronal activity or mGluRs activation can affect the subsequent ability to exhibit synaptic plasticity [for reviews, see 61,71-73]. Nevertheless, the current literature mainly indicates the metaplastic role of mGluRs in facilitation of hippocampal LTP induction, with less emphasis placed upon their effect on LTD in cortical areas [59,60,74,75]. There are only a few reports showing that priming stimulation of group II mGluRs inhibits or facilitates the subsequent induction of LTD in CA1 or dentate gyrus, respectively [76,77], while prior activation of group I mGluRs has no effect [77]. Our recent work in the ACC revealed a facilitatory role of prior mGluR1 activation on cingulate LTD induction in the tail-amputated mice [38]. Consistently, the present study demonstrated a similar rescue of amputation-impaired insular LTD by priming treatment with DHPG (20 μM, 20 min). This is the first demonstration of the metaplasticity phenomenon in the adult mouse IC. More importantly, these observations highlight the potential of developing mGluR agonists as a novel therapeutic strategy against phantom pain (see below).

**Intracellular protein kinases mediating the pharmacological rescue**

In the present study, we also examined the mechanisms of group I mGluR-mediated metaplastic rescue of insular LTD. Previously, evidence has been obtained to support the role of PKC in various types of metaplasticity,
NMDA receptor- or prior synaptic activity-induced subsequent LTP inhibition and LTD facilitation [63,78], mGluRs-mediated LTP enhancement [64,74,79] and inhibition of chemically- or electrically-induced LTD initiation [65,80]. Here, we have added the new findings that PKC activation is also an important contributing factor governing group I mGluR-mediated metaplastic rescue of amputation-impaired insular LTD. By contrast, we did not find any participation of PKA or CaMKII in the process, although there are some previous reports indicating their involvement in the regulation of meta-plasticity [66-68]. Consistently, our previous work found that amputation-induced loss of LTD in the ACC was rescued by mGluR1-related PKC-dependent mechanisms [38], suggesting the important roles of PKC in mGluR-evoked metaplasticity in both ACC and IC. It is well known that group I mGluR activation can lead to intracellular calcium rise and subsequent PKC activation [81,82]. Also, the function of the NMDA receptor can be regulated through PKC-mediated signaling pathways [74,83,84]. Recently, we reported that the NMDA receptor is involved in the induction of LFS-evoked LTD in the IC [44]. It is thus reasonable to speculate that bath application of DHPG might result in significant PKC activation, which then contributes to the restoration of insular LTD through possible NMDA receptor-related mechanisms in the IC slices from tail-amputated mice. Importantly, inhibition of PKC did not affect the LTD induction in naïve IC slices [44], implying that mechanistic differences do exist between synaptic plasticity and metaplasticity in the IC.

Clinical implications
Phantom pain is a common form of chronic pain syndrome characterized by the feeling of pain in the missing limb following amputation or deafferentation [25-27]. Until now, the clinical treatment for phantom pain is still limited and inefficient. Maladaptive plastic changes along the neuroaxis have been proposed to be associated with the occurrence and intensity of phantom pain [25,31,32,85]. Therefore, reversing these plastic changes may offer a novel way to improve the treatment of phantom pain or amputation-related brain dysfunctions. Our previous and present results reveal a loss of LFS-induced LTD in the ACC [38] and IC (the present study) following tail amputation in the adult mice, providing an alternative mechanism by which peripheral injury elicits long-lasting alterations in synaptic transmission and function in the central nervous system [37,42,47, also see Table 1]. Furthermore, we demonstrate that priming treatment with DHPG application could rescue the lost LTD in both ACC and IC after amputation, indicating that drugs acting at group I mGluRs might hold promise for the rational treatment of phantom pain by reversing amputation-evoked synaptic dysfunctions in the neocortex. From a clinical perspective, the multi-synaptic model established in the present study might be useful for further elucidating synaptic mechanisms of phantom pain in the brain, as well as screening and developing potential new drugs for treating this intractable disease in the human amputee.

Methods

Animals
Experiments were performed with adult (7-10 week old) male C57/BL6 mice purchased from Charles River (Quebec, Canada). All animals were fed in groups of three per cage under standard laboratory conditions (12 h light/12 h dark, temperature 22-26°C, air humidity 55-60%) with ad libitum water and mice chow. The experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Toronto. All animals were maintained and cared for in compliance with the guidelines set forth by the International Association for the Study of Pain [86]. The number of animals used and their suffering were greatly minimized.

Drugs
The drugs used in this study include: DHPG, chelerythrine, KT5720 and KN62. Among them, chelerythrine and DHPG were dissolved in distilled water, while KT5720 and KN62 were prepared in dimethyl sulfoxide (DMSO) as stock solutions for frozen aliquots at -20°C. All these drugs were diluted from the stock solutions to the final desired concentration in the artificial cerebrospinal fluid (ACSF) before immediate use. The diluted DMSO in ACSF had no effect on baseline synaptic transmission and plasticity. Chelerythrine, KT5720 and KN62 were purchased from Tocris Cookson (Bristol, UK) and DHPG was obtained from Abcam Biochemicals (Cambridge, UK). The doses for each compound were chosen based on our preliminary experiments and on relevant information from previous papers [38,44,77]. For the pharmacological rescue of insular LTD, DHPG (20 μM) with or without the drugs was bath applied for 20 min and then washed out for 30 min prior to LTD induction.

Tail amputation
The major procedures for tail amputation are in accordance with those described previously [38,45,87]. After anesthesia with gaseous isoflurane, the mouse was gently put in a box where a 2.5 cm length of the tail tip was removed using surgical scissors. A drop of Krazy Glue was used to stop bleeding. The mice typically recovered from anesthesia within 3-5 min. Amputated animals did not exhibit any neurological deficits or abnormal behaviors when returned to the home cage. For the sham control group, mice were anesthetized for the same period of time.
without any surgery. Procedure was executed with caution to minimize handling-induced stress in the mice. In the present study, we performed electrophysiological recordings at 2 weeks after tail amputation (Figure 1A), on the basis of our previous reports showing an evident plastic change in the ACC at this time point [37–39].

**Insular slice preparation**

The general procedures for making IC slices are similar to those described previously [18,23,44,48]. Briefly, mice were anesthetized with a brief exposure to gaseous isoflurane and decapitated. The entire brain was rapidly removed and immersed into a cold bath of oxygenated (95% O2 and 5% CO2) ACSF containing (in mM): NaCl 124, KCl 2.5, NaH2PO4 1.0, MgSO4 1, CaCl2 2, NaHCO3 25 and glucose 10, pH 7.35–7.45. After cooling for 1–2 min, appropriate portions of the brain were then trimmed and the remaining brain block was glued onto the ice-cold stage of a vibrating tissue slicer (Leika, VT1000S). Following this, three coronal IC slices (300 μm) were obtained at the level of corpus callosum connection and transferred to an incubation chamber continuously perfused with oxygenated ACSF at 26°C. Slices were allowed to recover for at least 2 h before any electrophysiological recording was started.

**Multi-channel field potential recordings**

A commercial 64-channel multi-electrode array system (MED64, Panasonic Alpha-Med Sciences, Japan) was used for extracellular field potential recordings in this study. Procedures for preparation of the MED64 probe and multi-channel field potential recordings were similar to those described previously [23,38,43,44]. The device had an array of 64 planar microelectrodes, each 50 × 50 μm in size, arranged in an 8 × 8 pattern (inter-electrode distance: 150 μm). Before use, the surface of the MED64 probe was treated with 0.1% polyethyleneimine (Sigma, St. Louis, MO, USA) in 25 mM borate buffer (pH 8.4) overnight at room temperature. After incubation, one slice was positioned on the MED64 probe in such a way that the IC area was entirely covered by the recording dish mounted on the stage of an inverted microscope (CKX41, Olympus). The relative location of the IC slice with the probe followed the anatomical atlas [88, also see Figure 1B]. Once the slice was settled, a fine mesh anchor (Warner Instruments, Harvard) was carefully disposed to ensure slice stabilization during recording. The slice was continuously perfused with oxygenated, fresh ACSF at the rate of 2–3 ml/min with the aid of a peristaltic pump (Minipuls 3, Gilson) throughout the entire experimental period.

After a 15–20 min recovery of the slice, one of the 64 available planar microelectrodes was selected from the 64-switch box for stimulation by visual observation through a charge-coupled device camera (DP70, Olympus) connected to the inverted microscope. For test stimulation, monopolar, biphasic constant current pulses (0.2 ms in duration) generated by the data acquisition software (Mobius, Panasonic Alpha-Med Sciences) were applied to the deep layer (layer V–VI) of the IC slice at 0.008 Hz (red dot in Figure 1B). The fEPSPs evoked at both the deep layer and the superficial layer (layer I–III) of the IC slice were amplified by a 64-channel amplifier, displayed on the monitor screen and stored on the hard disk of a microcomputer for offline analysis. After selecting the best stimulation site and stabilizing the baseline synaptic responses, an input–output curve was first determined for each group using the measurements of fEPSP slope or the number of activated channels (output) in response to a series of ascending stimulation intensities from 6 μA to 24 μA by every 2 μA step (input). For the LTD induction, the intensity of the test stimulus was adjusted to elicit 40–60% of the maximum according to the input–output curves. Stable baseline responses were then monitored for at least 20 min before delivering a classical LFS protocol (1 Hz, 900 pulses, with the same intensity as baseline recording) to induce NMDA receptor-dependent LTD. In another set of experiments, DHPG (100 μM, 20 min) was bath applied to induce another form of LTD [44]. After LFS or DHPG application, the test stimulus was repeatedly delivered once every 2 min for 1 h or 50 min to monitor the time course of insular LTD.

**Data analysis**

All multi-channel electrophysiological data were analyzed offline by the MED64 Mobius software. For quantification of the input–output relationship, the slope of fEPSP was measured and expressed as the percentage of 8 μA value according to different layers (superficial layer and deep layer). It is notable that data from the stimulation intensity of 6 μA were not included in the slope analysis due to the much fewer fEPSP evoked at this low intensity. The number of activated channels evoked at different stimulation intensities was also counted in a blind manner. For quantification of the LTD data, the initial slope of fEPSPs was measured by taking the rising phase between 10% and 90% of the peak response, normalized and presented separately in both superficial and deep layers as a percentage change from the baseline level. The degree of LTD in each experiment was shown as the value obtained at 50 min or 60 min after DHPG or LFS treatment, respectively. For evaluation of the drug effects on rescued LTD, the averaged value of the last 10 min of the recording was compared statistically. Furthermore, the number of activated channels (over 20% of baseline, i.e. the amplitude goes over ~20 μV) vs. the LTD-showing (depressed by at least 15% of baseline) channels was counted and expressed as the induction ratio of LTD (number of LTD-occurring channels /number of all activated channels × 100%). All data are shown as mean ±
S.E.M. When necessary, the statistical significance was assessed by two-tailed Student’s t and one-way ANOVA (followed by post doc Fisher’s LSD test) using the Sigma Plot software. P < 0.05 was assumed as statistically significant.

Abbreviations

ACC: Anterior cingulate cortex; ACSF: Artificial cerebrospinal fluid; CaMKII: Calcium/calcmodulin-dependent protein kinase II; DHPG: (RS)-3,5-dihydroxyphenylglycine; DMSO: Dimethyl sulfoxide; fEPSP: Field excitatory postsynaptic potential; IC: Insular cortex; LFS: Low-frequency stimulation; LTD: Long-term depression; LTP: Long-term potentiation; MED64: 64-channel multi-electrode dish; mGluR: Metabotropic glutamate receptor; PKA: Protein kinase A; PKC: Protein kinase C.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

M-G.L. performed the experiments, analyzed data and drafted the manuscript; M.Z. conceived and designed the research and finished the final version of the manuscript. Both authors read and approved the final manuscript.

Acknowledgements

This work was supported by Canadian Institutes of Health Research (CIHR) operating grant, Canada Research Chair (CRC), and NSERC (Natural Sciences and Engineering Research Council of Canada) discovery grant 402555 to MZ.

Received: 1 November 2013 Accepted: 30 December 2013 Published: 8 January 2014

References

1. Craig AD: How do you feel–now? The anterior insula and human awareness. Nat Rev Neurosci 2009, 10(1):59–70.
2. Gal-Ben-Ari S, Rosenblum K: Molecular mechanisms underlying memory consolidation of taste information in the cortex. Front Behav Neurosci 2012, 6:87.
3. Agapian AV, Baliki MN, Ghea PY: Towards a theory of chronic pain. Prog Neurobiol 2009, 87(2):81–97.
4. Henderson LA, Gandevia SC, Macefield VG: Phantom limb pain and bodily awareness: current concepts and future directions. Curr Opin Anaesthesiol 2011, 24(4):524–531.
5. Flor H, Nikolajsen L, Stahlberg E, Jensen T: Maladaptive CNS plasticity? J Neurosci 2006, 26(6):1363–1368.
6. Scharenberg M, Siemssen F, Unterrainer S, Wolters C, Fleischer S, Seifert V, Nentwich A, Schmelz M: The unpleasantness of tonic pain is encoded by the insular cortex. Neurology 2005, 64(7):1175–1183.
7. Majiozo L, Lisic N, Malacarne L, Bajec B, Ljubisavljevic P, Modic M: Plasticity in the rat that contributes to morphine antinociception. J Neurosci 1999, 19(21):RC36.
8. Majozi L, Lisic N, Malacarne L, Bajec B, Ljubisavljevic P, Modic M: Somatosensory cortex in the agranular insular cortex of the rat that contributes to morphine antinociception. J Neurosci 1999, 19(21):RC36.
9. Flor H, Nikolajsen L, Stahlberg E, Jensen T: Phantom limb pain: a case of maladaptive CNS plasticity? Nat Rev Neurosci 2006, 7(11):873–881.
10. Flor H: Maladaptive plasticity, memory for pain and phantom limb pain: review and suggestions for new therapies. Expert Rev Neurother 2008, 8(5):809–818.
11. Giurumaru MJ, Moseley GL: Phantom limb pain and bodily awareness: current concepts and future directions. Curr Opin Anaesthesiol 2011, 24(4):524–531.
12. Flor H: Maladaptive plasticity, memory for pain and phantom limb pain: review and suggestions for new therapies. Expert Rev Neurother 2008, 8(5):809–818.
13. Flor H, Nikolajsen L, Stahlberg E, Jensen T: Phantom limb pain: a case of maladaptive CNS plasticity? Nat Rev Neurosci 2006, 7(11):873–881.
14. Flor H: Maladaptive plasticity, memory for pain and phantom limb pain: review and suggestions for new therapies. Expert Rev Neurother 2008, 8(5):809–818.
15. Flor H, Nikolajsen L, Stahlberg E, Jensen T: Phantom limb pain: a case of maladaptive CNS plasticity? Nat Rev Neurosci 2006, 7(11):873–881.
16. Flor H, Nikolajsen L, Stahlberg E, Jensen T: Phantom limb pain: a case of maladaptive CNS plasticity? Nat Rev Neurosci 2006, 7(11):873–881.
17. Flor H, Nikolajsen L, Stahlberg E, Jensen T: Phantom limb pain: a case of maladaptive CNS plasticity? Nat Rev Neurosci 2006, 7(11):873–881.
Pain-related increase of excitatory transmission

36. Li J, Wu M, Zhuo M, Xu ZC. Alteration of neuronal activity after digit amputation in rat anterior cingulate cortex. Int J Physiol Pathophysiol Pharmacol 2013, 5(1):143–51.

37. Wei F, Li P, Zhuo M. Loss of synaptic depression in mammalian anterior cingulate cortex after amputation. J Neurosci 1999, 21(8):3936–9354.

38. Kang S, Liu MG, Chen T, Ko HG, Baek GC, Lee HR, Lee K, Collingridge GL, Kang BK, Zhuo M. Plasticity of metabotropic glutamate receptor-dependent long-term depression in the anterior cingulate cortex after amputation. J Neurosci 2012, 32(33):11318–11329.

39. Wei F, Zhuo M. Activation of Erk in the anterior cingulate cortex during the induction and expression of chronic pain. Mol Pain 2008, 4(2):42.

40. Willoch F, Rosen G, Tolle TR, Oye I, Wester HJ, Berner N, Schwaiger M, Bartenstein P. Phantom limb pain in the human brain: unraveling neural circuits of phantom limb sensations using positron emission tomography. Ann Neurol 2000, 48(6):842–849.

41. Rosen G, Hugdahl K, Erlanson L, Lundervold A, Smievoll AI, Bavand R, Sundberg H, Thomsen T, Roscher BE, Tjolsen A, et al. Different brain areas activated during imagery of painful and non-painful ‘finger movements’ in a subject with an amputated arm. Neuroscience 2001, 7(3):255–260.

42. Zhuo M. Cortical depression and potentiation: basic mechanisms for phantom pain. Exp Neurol 2012, 214(1):129–135.

43. Li XY, Ko HG, Chen T, Descalzi G, Koga K, Wang H, Kim SS, Shang Y, Kwak C, Park SW, et al. Alleviating neuropathic pain hypersensitivity by inhibiting PKMζ in the anterior cingulate cortex. Science 2010, 330(6009):1400–1404.

44. Liu MG, Koga K, Guo YY, Kang SJ, Collingridge GL, Kaang BK, Zhao MG, Zhuo M. Long-term depression of synaptic transmission in the adult mouse insular cortex in vitro. Eur J Neurosci 2013, 38(8):3128–3145.

45. Wei F, Xu ZC, Qu Z, Milbrandt J, Zhuo M. Role of EGR1 in hippocampal synaptic enhancement induced by tetanic stimulation and amplification. J Cell Biol 2003, 164(4):1325–1334.

46. Kodama D, Ono H, Tatsube M. Altered hippocampal long-term potentiation after peripheral nerve injury in mice. Eur J Pharmacol 2007, 574(2–3):127–132.

47. Chou CS, Huang CC, Liang YC, Tai YC, Hsu KS. Impairment of long-term depression in the anterior cingulate cortex of mice with bone cancer pain. Pain 2012, 153(3):2097–2108.

48. Koga K, Sim SE, Chen T, Wu LJ, Kaang BK, Zhuo M. Kainate receptor-mediated synaptic transmissions in the adult rodent insular cortex. J Neurophysiol 2012, 107(8):1988–1998.

49. Neuvonen YR. The insular cortex: a review. Prog Brain Res 2012, 195:253–123.

50. Jasmin L, Burkey AR, Granato A, Obara PT. Rostral agranular insular cortex and pain areas of the central nervous system: a tract-tracing study in the rat. J Comp Neurol 2004, 468(3):425–440.

51. Zhao MG, Ko SW, Wu LJ, Toyoda H, Xu H, Quan L, Ji L, Jia Y, Ren M, Xu ZC, et al. Enhanced presynaptic neurotransmitter release in the anterior cingulate cortex of mice with chronic pain. J Neurosci 2006, 26(35):8923–8930.

52. Xu H, Wu LJ, Wang H, Zhang X, Vadakkan KJ, Kim SS, Steenland HW, Zhuo M. Presynaptic and postsynaptic amplifications of neuropathic pain in the anterior cingulate cortex. J Neurosci 2008, 28(29):7445–7453.

53. Toyoda H, Zhao MG, Zhuo M. Enhanced quantal release of excitatory transmitter in anterior cingulate cortex of adult mice with chronic pain. Mol Pain 2009, 5(4).

54. Gong KR, Cao FL, He Y, Gao CY, Wang DO, Li H, Zhang FK, An YY, Lin Q, Chen J. Enhanced excitatory and reduced inhibitory synaptic transmission contribute to persistent pain-induced neuronal hyper-responsiveness in anterior cingulate cortex. Neuroscience 2010, 171(1):1314–1325.

55. Neugebauer V, Li W, Bird GC, Bhave G, Gereau RW. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J Neurosci 2003, 23(15):52–63.

56. Ji G, Sun H, Fu Y, Li Z, Pais-Veira M, Galhardo V, Neugebauer V. Cognitive impairment in pain through amygdala-driven prefrontal cortical deactivation. J Neurosci 2010, 30(15):5451–5464.

57. Kang W, Neugebauer V. Plasticity of metabotropic glutamate receptors. Mol Pain 2010, 6:93.

58. Zhao XY, Liu MG, Yuan DL, Wang Y, He Y, Wang DO, Chen XF, Zhang FK, Li H, He XS, et al. Nociception-induced spatial and temporal plasticity of synaptic connection and function in the hippocampal formation of rats: a multi-electrode array recording. Mol Pain 2009, 5:555.
81. Neugebauer V. Metabotropic glutamate receptors: novel targets for pain relief. Expert Rev Neurother 2001, 1(2):207–224.
82. Neugebauer V, Carlton SM. Peripheral metabotropic glutamate receptors as drug targets for pain relief. Expert Opin Ther Targets 2002, 6(3):349–361.
83. Li YC, Liu G, Hu JL, Gao WJ, Huang YQ. Dopamine D(1) receptor-mediated enhancement of NMDA receptor trafficking requires rapid PKC-dependent synaptic insertion in the prefrontal neurons. J Neurochem 2010, 114(1):62–73.
84. Slack SE, Pezet S, McMahon SB, Thompson SW, Malcangio M. Brain-derived neurotrophic factor induces NMDA receptor subunit one phosphorylation via ERK and PKC in the rat spinal cord. Eur J Neurosci 2004, 20(7):1769–1778.
85. Jones EG. Thalamic and brainstem contributions to large-scale plasticity of primate somatosensory cortex. Science 1998, 282(5391):1121–1125.
86. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983, 16(2):109–110.
87. Zhuo M. NMDA receptor-dependent long term hyperalgesia after tail amputation in mice. Eur J Pharmacol 1998, 349(2-3):211–220.
88. Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates. San Diego: Academic Press, 2001.