Potentiation of sensory responses in the anterior cingulate cortex following digit amputation in the anaesthetised rat

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1. The anterior cingulate cortex (ACC) is important for processing different types of information, including sensory inputs. In the present study on anaesthetised rats, we recorded *in vivo* sensory responses of the ACC to peripheral electrical shocks. Peripheral electrical stimulation at high intensities sufficient to activate nociceptive sensory fibres elicited EPSPs within the ACC.

2. Digit amputation caused long-lasting potentiation of ACC responses to peripheral electrical stimulation. Evoked field EPSPs remained enhanced for at least 120 min after the amputation. Because electrical shocks were delivered to the normal hindpaw, it is likely that plastic changes occur centrally in the spinal cord or the supraspinal structures following amputation.

3. We also recorded field EPSPs of the ACC in response to focal cortical stimulation within the ACC. Like the sensory responses, field EPSPs produced by focal cortical stimulation within the ACC were potentiated after digit amputation, suggesting that long-lasting changes occurred locally within the ACC.

4. Local blockade of peripheral activity by QX-314 at the amputated hindpaw 120 min after amputation did not significantly affect sensory responses induced within the ACC. Thus, peripheral ongoing inputs do not play an important role in maintaining potentiation within the ACC.

5. Two pulses of hindpaw stimulation caused paired-pulse depression in the ACC. Local stimulation within the ACC also caused depression of sensory responses to hindpaw stimulation, suggesting that the population of synapses activated by local stimulation may overlap with that activated by peripheral hindpaw stimulation.

6. Our results suggest that rapid enhancement of sensory responses can be observed in the ACC after amputation and that enhanced neuronal responses to subsequent somatosensory stimuli may contribute to phantom-limb pain.

The adult somatosensory cortex is dynamic and plastic (Kaas, 1991; Ramachandran, 1993; Gilbert, 1996; Buonomano & Merzenich, 1998). Instead of being stable and constant throughout life, recent studies indicate that cortical anatomic connections as well as functional representations can be modified by experience. These modifications can occur within a very short amount of time. Such changes occur not only during development and under normal physiological conditions, but also under pathological conditions, such as tissue/nerve injury or the loss of a limb. It has been demonstrated that cortical reorganisation occurs after limb or digit amputation (Merzenich *et al.* 1984; Pons *et al.* 1991; Ramachandran *et al.* 1992, 1995; Florence *et al.* 1998; Jones & Pons, 1998; Kaas, 1998). Most human amputees experience phantom limb sensation or phantom pain (Sherman *et al.* 1980; Melzack, 1990; Jensen & Rasmussen, 1994). The amount of cortical reorganisation correlates with the extent of phantom pain (Flor *et al.* 1995). However, molecular and cellular mechanisms contributing to plastic changes in the neocortex after amputation remain to be investigated.

The anterior cingulate cortex (ACC) forms a large region around the rostrum of the corpus callosum, and it responds to nociceptive stimuli in animals (Sikes & Vogt, 1992) and painful stimuli in humans (Talbot *et al.* 1991; Vogt *et al.* 1996; Davis *et al.* 1997; Derbyshire *et al.* 1998; Lenz *et al.* 1998). It has been proposed that activity in the ACC may underlie the pleasantness or discomfort associated with some somatosensory stimuli. Consistently, for patients with lesions in the ACC, it has been reported that chronic pain is reduced (Yarnitsky *et al.* 1988).

Excitatory synaptic transmission within the ACC is mediated by the fast neurotransmitter glutamate (Sah & Nicoll, 1991; Tanaka & North, 1994; Wei *et al.* 1999). Evoked EPSCs recorded using intracellular or whole-cell patch-clamp techniques were mediated through post-synaptic AMPA/kainate receptors (Sah & Nicoll, 1991; Tanaka & North, 1994; Wei *et al.* 1999). In addition, opioids inhibited EPSPs/EPSCs in the ACC, indicating that the regulation of neurotransmission within the ACC may contribute to the antinociceptive/analgesic effects of opioids (Tanaka & North, 1994; Wei *et al.* 1999). Consistently, in awake animals, local administration of different opioid receptor agonists in the ACC produced powerful antinociceptive effects (Lee *et al.* 1999). Furthermore, *in vitro* electrophysiological studies on cortical slices showed that glutamate synapses within the ACC undergo activity-dependent plastic changes (Sah & Nicoll, 1991; Wei *et al.* 1999).

Studies using *in vitro* cortical slices have provided basic information about excitatory transmission as well as plastic changes after amputation (Wei *et al.* 1999). It is important to determine whether plastic changes could occur under more physiological conditions such as in intact animals. In the present study, we performed *in vivo* electrophysiological experiments on adult rats to examine: (1) whether peripheral noxious electrical shocks can induce sensory responses in the ACC in lightly anaesthetised adult rats; (2) whether these responses are altered after amputation of a single digit; and (3) whether long-lasting changes in responses of the ACC to peripheral shocks are at least in part due to local synaptic changes within the ACC.

METHODS

Animals

Adult male Sprague-Dawley rats weighing 250–280 g (Harlan, Indianapolis, IN, USA) were used for all experiments. Animals were housed in an animal care facility with a 12 h light–12 h dark cycle and with food and water *ad libitum*. The experimental protocols were approved by the Animal Studies Committee at Washington University. A light level of anaesthesia (corneal, auricular-pinnal and flexion reflexes present) was maintained by continuous application of halothane through a gas anaesthesia adaptor (Stoelting, Wood Dale, IL, USA).

In vivo electrophysiology

Rats were anaesthetised with 2–3 % halothane in a gas mixture of 30 % O_2 balanced with nitrogen. A craniotomy was performed in the region overlying the ACC and frontal cortex, and the dura was cut and removed from the exposed area of cortex. Animals were subsequently maintained at around 1 % halothane. All wound margins were covered with a local anaesthetic ointment (Nupercainal, Rugby Laboratories, Inc., Norcross, GA, USA), and the rats were placed in a stereotaxic apparatus. Body temperature was maintained at 37 °C using a thermostatically regulated heating blanket. A bipolar electrode was inserted subcutaneously into one hindpaw. Extracellular field EPSPs were recorded from the ACC contralateral or ipsilateral to the amputated digit with a glass microelectrode filled with 4 % Neurobiotin (Vector Laboratories, Inc., Burlingame, CA, USA) solution with 2 M potassium acetate in artificial cerebrospinal fluid (ACSF, pH 8.0). The depth (about 1–2 mm) of the recording electrodes was determined according to the shape of the evoked responses. Responses were evoked at 0.01 Hz. The intensity for peripheral stimulation eliciting synaptic responses in the ACC ranged from 10 to 100 V (0.5 ms). The latency for field EPSPs was independent of the stimulation intensity. The slope of the field EPSPs was measured using the pCLAMP 5.0 program (Axon Instruments, Inc., Foster City, CA, USA).

To determine whether synaptic changes may happen locally within the ACC, we recorded synaptic responses of the ACC to focal electrical stimulation within the ACC. A bipolar tungsten electrode was placed within the ACC area on the same side of the cortex as the recording electrode. Test stimuli (0.2 ms duration) were applied every 50 s.

In some experiments, paired-pulse stimulation was used to determine whether synaptic responses induced by local ACC stimulation may also be activated by sensory inputs from the hindpaw. Two pulses of stimulation were delivered with a 50 ms interval. For hindpaw stimulation, the synaptic response to the second pulse was significantly depressed (which is different from that in the CA1 region of the hippocampal slices). We then replaced the first hindpaw stimulation with the ACC stimulation to see whether the response to the hindpaw stimulation was affected.

To examine the roles of peripheral activity in ACC responses, two different types of experiment were performed: (1) reversible blockade of peripheral nerve activity by local injection of an anaesthetic, QX-314, into the hindpaw (5 %, 50 μl) or the third digit (5 %, 25 μl) of the hindpaw; (2) high-frequency stimulation (100 Hz, 1 s; single or twice with a 20 s interval) at the same stimulation intensity delivered to the third digit of the hindpaw.

Digit amputation

After stable baseline responses had been obtained for at least 30 min, anaesthesia was deepened with 2–3 % halothane. The third digit of the unstimulated hindpaw was amputated (see Merzenich *et al.* 1984; Wei *et al.* 1999). Any bleeding was stopped with cyanoacetate. After 2–3 min, the level of halothane was reset to 1 %. In the sham-operated animals, the same change in anaesthesia was made, but the digit was not removed. The synaptic responses to peripheral stimulation in both the sham-treated and amputee animals were completely inhibited by intraperitoneal administration of morphine (10 mg kg^{-1}; authors' unpublished data). Considering the intensity of stimulation and the sensitivity to morphine, it is likely that nociceptive Aδ and C fibres were activated. Activation of the ACC as well as the somatosensory cortex has been reported in humans by painful electrical nerve stimulation (Davis *et al.* 1997).

Histological identification of recording sites

On completion of the experiment, identification of the recording site was carried out via the neurobiotin-filled electrodes using a depolarising pulse (5–10 nA, 200 ms, 1 Hz, 10 min). Then the animal was deeply anaesthetised and perfused transcardially with saline, followed by 4 % paraformaldehyde. Serial cryostat sections (30 μm) of the ACC were incubated in peroxidase-conjugated avidin–biotin complex (ABC, 1:100, Vector Laboratories) and stained with 3,3′-diaminobenzidine and hydrogen peroxide. Cresyl violet was used as a counterstain. Recording sites from individual experiments were identified on coronal ACC sections based on the atlas of Paxinos & Watson (1986). As shown in the representative section in Fig. 1*B*, all recording sites were located in the ACC areas.

Data and analysis

Data are presented as the mean ± S.E.M. Statistical comparisons were made by analysis of variance (ANOVA; Newman-Keuls tests for *post hoc* comparison) or Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Recording EPSPs evoked by peripheral electrical shock or focal ACC stimulation in anaesthetised rats

Glutamate is the major excitatory transmitter in the central nervous system (CNS). In the ACC, postsynaptic AMPA/kainate receptors mediate fast synaptic currents (Tanaka & North, 1994; Wei *et al.* 1999). Application of CNQX completely blocked synaptic responses (Wei *et al.* 1999). To measure synaptic responses to peripheral electrical shocks, we placed a recording electrode in the ACC of anaesthetised rats (Fig. 1*A*). Experiments were performed at least 30–60 min after preparing the animals. Electrical stimulation at different intensities was delivered to one hindpaw at 50 s intervals. At high intensities of stimulation, sufficient to activate Aδ and C fibres, evoked field EPSPs were found in the ACC. Evoked responses were intensity dependent. At low intensities of stimulation, few or no responses were observed (see Fig. 1*C* for an example). Due to potential variations in tissue halothane concentrations between experiments, we felt that the measurement of a threshold for inducing field EPSPs was not reliable. Indeed, field EPSPs were sensitive to halothane; field

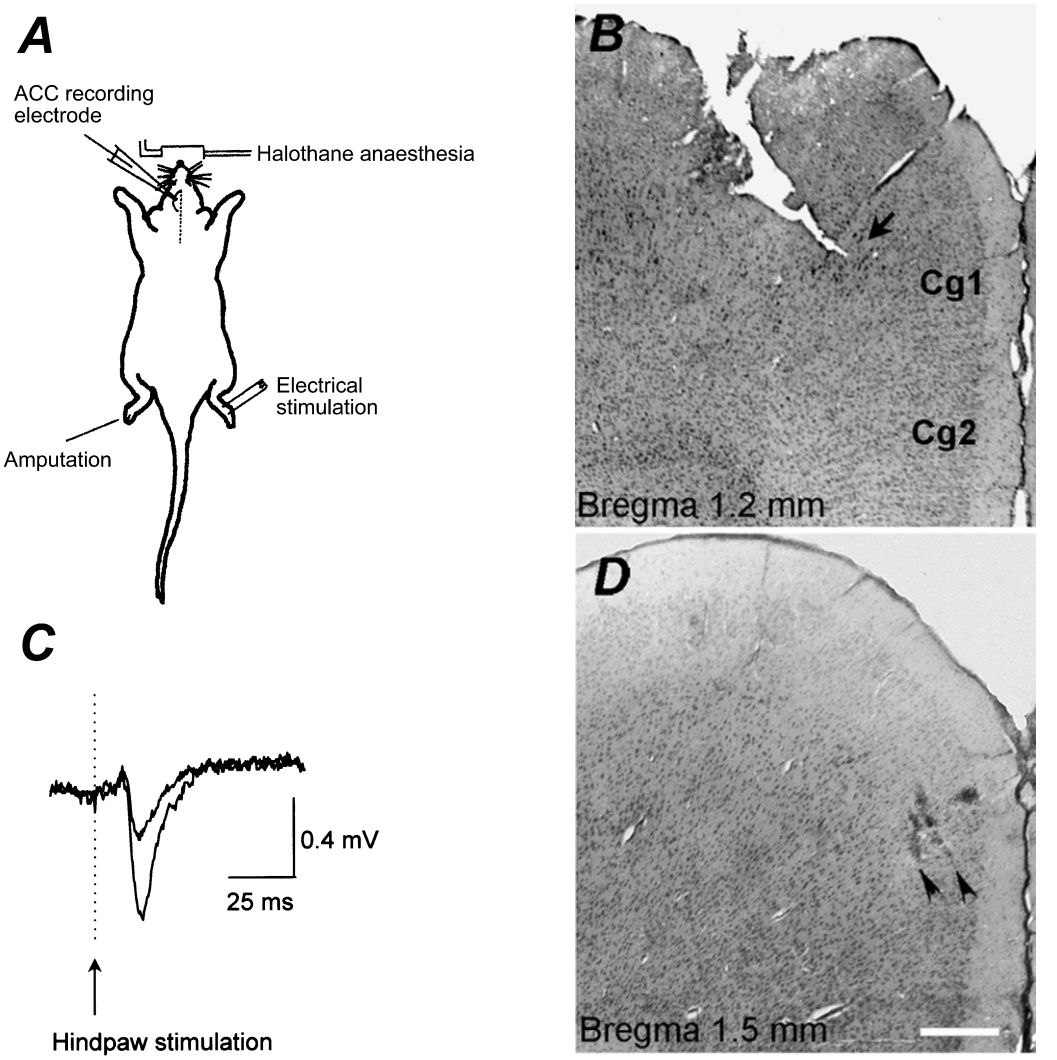

Figure 1. Sensory response of the anterior cingulate cortex to peripheral stimulation in adult rats

A, diagram of *in vivo* recording from the anterior cingulate cortex (ACC) in an anaesthetised rat; animals were maintained in a lightly anaesthetised state by halothane. The recording electrode was placed into the ACC contralateral to the peripheral stimulation electrode. Amputation (the removal of the third digit of the hindpaw) was performed on the non-stimulated hindpaw. During amputation, a higher concentration of halothane was used. *B*, example of a histological section showing the recording site (arrow) labelled with neurobiotin and the track of the recording electrode. *C*, traces of synaptic responses to electrical stimulation applied to the hindpaw at a low intensity (5.0 V) and a higher intensity (25.0 V). An arrow indicates the time of hindpaw electrical stimulation. *D*, example of a histological section showing the ACC focal bipolar stimulating sites (arrowheads). Scale bar is 300 μm for *B* and *D*. Abbreviations: Cg1, cingulate cortex, area 1; Cg2, cingulate cortex, area 2.

EPSPs were depressed when a higher dose of halothane was applied.

The field EPSPs recorded from the ACC were obviously polysynaptic in nature, probably involving at least primary afferent fibres and spinothalamic and thalamocortical tracts. Nevertheless, we estimated the response latency between the start of stimulation and the onset of field EPSPs. The mean latency for the onset of field EPSPs was 12.0 ± 0.1 ms ($n = 6$).

Long-lasting enhancement caused by amputation

Peripheral tissue/nerve injury causes central sensitisation. These long-lasting changes could occur at different levels of the CNS, including the spinal cord, brainstem, thalamus and cortex (see Introduction). Previous studies found that amputation of a central digit of the hindpaw caused long-lasting changes in the ACC: (1) different immediate-early genes (IEGs) were activated in the ACC; and (2) long-term synaptic depression recorded from *in vitro* cingulate slices of amputated rats was significantly decreased or abolished. In the present study we wanted to find out, in an *in vivo* setting, whether sensory responses of the ACC to peripheral electrical shocks are also affected after amputation. Because amputation caused local damage to skin as well as nerves innervating the digit, we performed amputation on the hindpaw contralateral to the one to which stimulation was delivered. Therefore, any changes in synaptic responses were not simply due to the alteration of peripheral excitability caused by amputation.

Interestingly, after amputation of a central digit of the hindpaw, we observed a rapid enhancement of sensory responses to peripheral electrical shocks delivered to the normal hindpaw (Fig. 2). The potentiation was long lasting; evoked responses remained enhanced for at least 120 min. Field EPSPs were increased to a mean of $179.7 \pm 20.7\%$ of control at 110–120 min after amputation ($P < 0.05$ compared with the EPSP before the stimulation; Figs 3 and 4). In contrast, in animals receiving sham treatment, field EPSPs were not significantly affected at 120 min after the treatment ($111.3 \pm 4.8\%$, $n = 3$; Fig. 2). The enhancement observed in rats following amputation was significantly greater than that in sham-treated animals ($P < 0.05$).

Local synaptic potentiation within the ACC

In order to address the possibility that synaptic changes may occur locally within the ACC, we measured field EPSPs in response to focal ACC electrical stimulation delivered through a bipolar tungsten electrode (see Figs 1*D* and 3). Evoked field EPSPs had a much shorter latency to onset as compared to those evoked by hindpaw stimulation (Fig. 3). These field EPSPs recorded *in vivo* were similar to field EPSPs recorded from *in vitro* ACC slices (see Wei *et al.* 1999).

It was important to determine whether the increases in synaptic responses to peripheral electrical stimulation after amputation were due to local synaptic changes within the ACC or other central areas that convey sensory inputs to the ACC. In preliminary experiments,

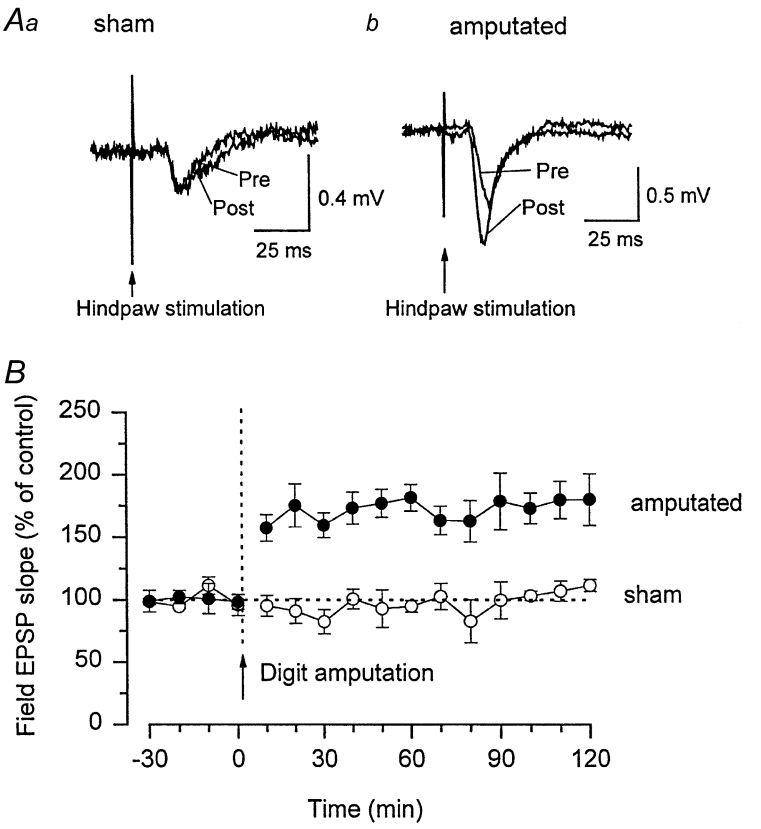

Figure 2. Long-lasting enhancement following amputation of a single hindpaw digit

A, representative traces of EPSPs 5 min before amputation (Pre) and 115–120 min after (Post) sham treatment (*a*) or amputation (*b*). In *b*, the latency of sensory responses was not changed after the amputation, while the EPSP slope was increased. *B*, amputation of a single digit of the contralateral hindpaw (indicated by an arrow; see Fig. 1) caused long-lasting enhancement of sensory responses (●). Sensory responses were not significantly changed in sham-treated animals (○). The test stimulation frequency was 0.01 Hz.

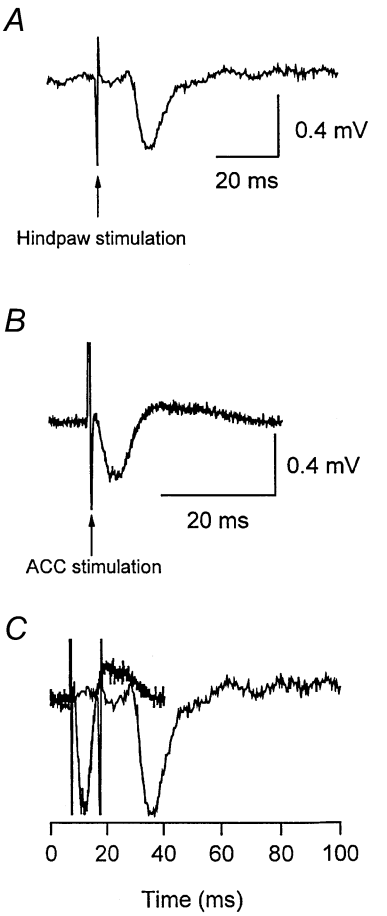

Figure 3. Sensory response of the ACC to focal electrical stimulation within the ACC in adult rats

A, a single trace of the response to electrical stimulation applied to the hindpaw. *B*, a single trace of the response to electrical stimulation applied within the ACC. *C*, the two traces from *A* and *B* were normalised to peak amplitude and are shown superimposed.

we placed the recording electrodes into the somatosensory cortex or the thalamus. However, we did not obtain any obvious field EPSPs with a clearly constant latency. Therefore, we recorded field EPSPs from the ACC evoked by local electrical stimulation. If amputation of a single digit altered synaptic plasticity within the ACC, we would expect to observe a similar long-lasting potentiation of field EPSPs. Indeed, we observed a long-lasting potentiation of field EPSPs after amputation and the enhancement lasted for at least 90 min ($n = 7$;

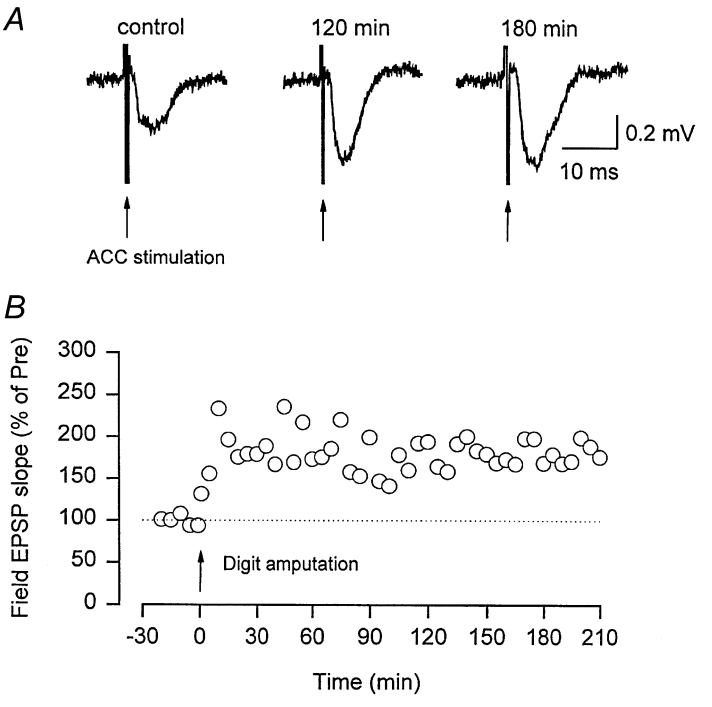

Figure 4. Long-lasting enhancement of sensory responses within the ACC following amputation of a single hindpaw digit

A, representative traces of EPSPs 5 min before amputation, and 115–120 and 175–180 min after amputation. *B*, a plot of field EPSP slopes (as a percentage of the pre-amputation value) over time for the example shown in *A*.

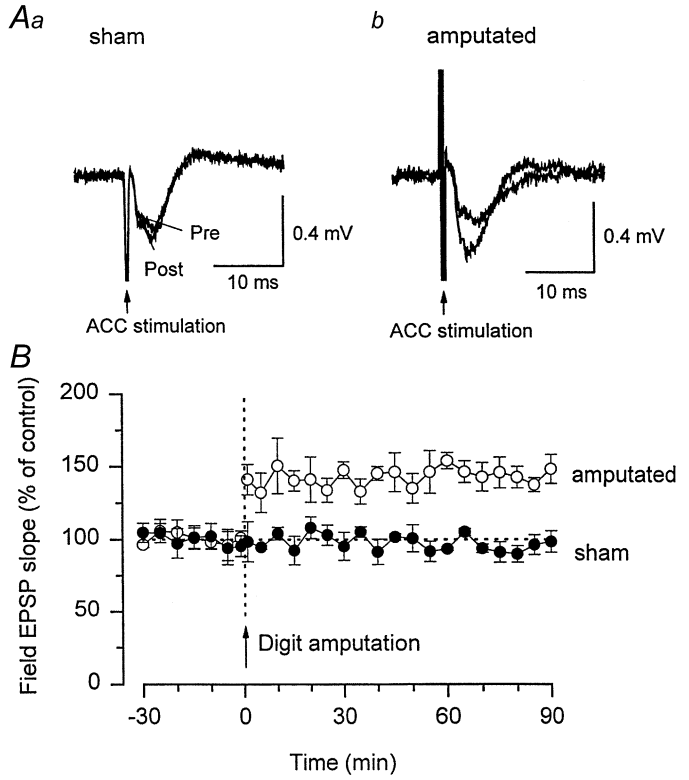

Figure 5. Amputation caused long-lasting enhancement of sensory responses within the ACC

A, representative traces of EPSPs 5 min before amputation and 85–90 min after sham treatment (*a*) or amputation (*b*). *B*, amputation of a single digit of the contralateral hindpaw (see Fig. 1) caused long-lasting potentiation of synaptic responses within the ACC (○). Sensory responses were not significantly changed in sham-treated animals (●). The test stimulation frequency was 0.02 Hz.

142.8 ± 6.3% between 85 and 90 min after amputation). In six of seven experiments, we continued the recording for up to 120 min after amputation and responses were still enhanced ($n = 6$; 149.6 ± 13.1% between 115 and 120 min after amputation; see Fig. 4 for an example). In contrast, in animals receiving sham treatment, field EPSPs were not significantly affected after the treatment (111.3 ± 4.8%, $n = 3$; Fig. 5). The synaptic enhancement observed in rats following amputation was significantly greater than that in sham-treated animals ($P < 0.05$). The amount of potentiation was not significantly different from that in field recordings evoked by hindpaw stimulation.

Peripheral ongoing activity is not required for potentiation

We hypothesise that long-lasting potentiation within the ACC is probably due to abnormal activities during and after amputation. One important question is whether potentiated sensory responses required persistent activity from the injured hindpaw. To test this, we locally injected an anaesthetic, QX-314, into the hindpaw (5%, 50 μl) 120 min after amputation. Our previous behavioural studies in adult rats showed that QX-314 significantly decreased nociceptive behavioural responses for at least 10–15 min post-injection (see Calejesan *et al.* 1998). Interestingly, we found that QX-314 injection did not significantly affect synaptic potentiation ($n = 4$; 153.4 ± 17.8% of control 0–5 min before *vs.* 148.8 ± 11.4% of control 5–10 min after QX-314 injection; Fig. 6).

Intra-ACC responses are linked to responses induced by peripheral stimulation

To examine whether local ACC stimulation-induced synaptic responses may overlap with hindpaw stimulation-induced responses, we performed experiments using a paired-pulse protocol. First, we measured evoked responses to two pulses of hindpaw stimulation delivered with a 50 ms interval. We found that the second responses were significantly decreased in comparison to the first

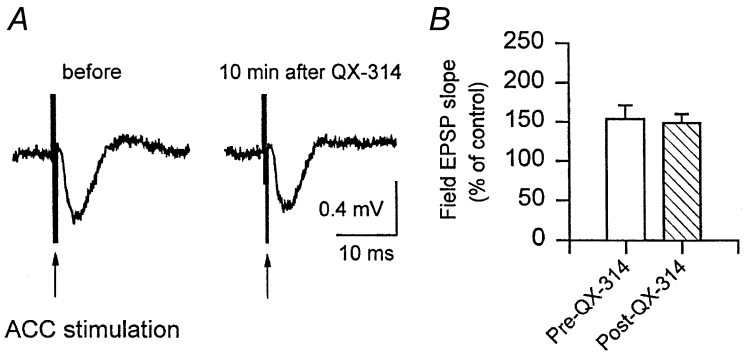

Figure 6. Persistent peripheral activity is not required for synaptic potentiation within the ACC following amputation of a single hindpaw digit

A, traces of sensory responses to electrical stimulation applied to the ACC before and 10 min after peripheral injection of QX-314. *B*, summarized data from the experiments shown in *A*.

responses ($n = 6$; $41.3 \pm 10.2\%$ of the first response, $P < 0.05$; see Fig. 7*A*). Interestingly, an initial pulse of local ACC stimulation also caused decreases in the synaptic responses to hindpaw stimulation delivered 50 ms later (Fig. 7*B*). Compared to responses obtained after an isolated pulse of hindpaw stimulation, sensory responses to hindpaw stimulation 50 ms after a pulse of local ACC stimulation were decreased significantly by $50.0 \pm 7.5\%$ ($n = 7$, $P < 0.05$). There was no significant difference in paired-pulse depression when the first pulse was delivered to the hindpaw (Fig. 7*A*) or locally in the ACC (Fig. 7*B*).

Brief silencing or stimulating peripheral inputs at a single digit

To examine whether local reversible deafferentation affects hindpaw stimulation-induced ACC responses, we locally injected QX-314 into the third digit of the unstimulated hindpaw (5%, 25 μl) after obtaining at least 10 min stable baseline responses. ACC responses to stimulation delivered to the contralateral hindpaw were not significantly affected ($n = 3$, see Fig. 8*A*). We also tested whether high-frequency stimulation of the third digit briefly affected ACC responses. Electrical stimulation was delivered at the same intensity as the test stimulus as either one train (100 Hz, for 1 s; $n = 4$) or two trains (100 Hz, 1 s twice with a 20 s interval; $n = 3$). As shown in Fig. 8*B* and *C*, no significant change in baseline responses was observed.

DISCUSSION

We report here that amputation of a single digit of one hindpaw caused rapid and prolonged changes in sensory responses within the ACC. Enhanced excitatory transmission in the ACC, a region critical for processing pain information in the CNS, may serve as an important synaptic mechanism for enhanced nociception after tissue and nerve injury. Because the increases in the ACC responses were very rapid (within a few minutes), we think that it clearly differs from somatosensory map reorganisation after amputation (see Introduction). It remains possible that these early changes may contribute to late cortical reorganisation. In the present studies, ACC responses to hindpaw stimulation were polysynaptic. It is very difficult to distinguish the true plasticity at the excitatory ACC synapses from possible disinhibition of inhibitory circuits under *in vivo* conditions. Thus, these early changes in the ACC may represent the immediate unmasking effects after amputation (e.g. unmasking of latent connections by disinhibition) and/or potentiation of excitatory glutamate synapses. Further experiments

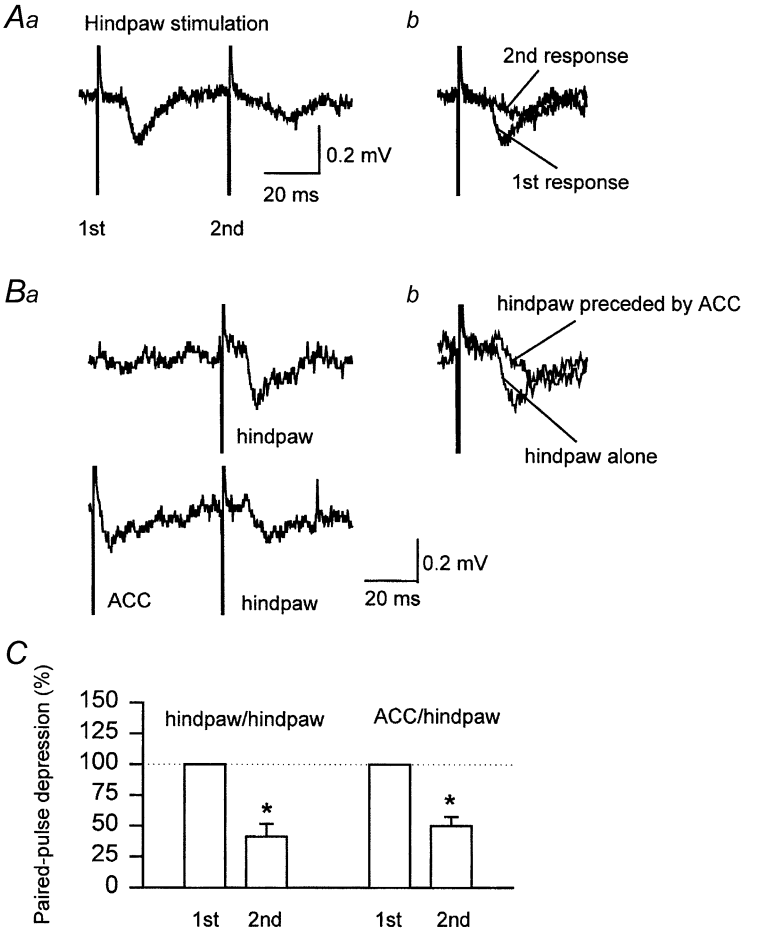

Figure 7. Paired-pulse depression induced by hindpaw or ACC stimulation

A, paired-pulse depression induced by two pulses of stimulation delivered to the hindpaw at the same intensity (*a*). In *b*, the two synaptic responses from *a* are superimposed for comparison. Results from six experiments are summarized in *C*. *B*, applying ACC local stimulation (50 ms before) depressed synaptic responses to hindpaw stimulation. In *a*, the upper trace shows a field response to hindpaw stimulation alone; the lower trace shows that ACC local stimulation induced a fast field response and depressed subsequent responses to the same hindpaw stimulation (paired-pulse depression). Traces of field synaptic responses are superimposed in *b* for comparison. Results from seven experiments are summarized in *C*. *C*, summarized results for experiments shown in *A* and *B*. * $P < 0.05$ compared to the first response.

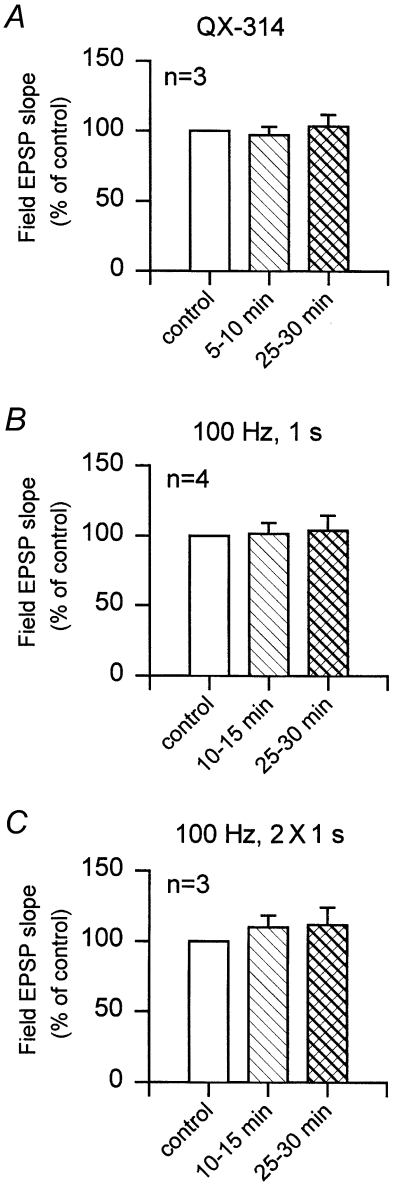

Figure 8. Brief silencing or stimulating peripheral inputs at a single digit

A, ACC responses to hindpaw stimulation were not affected by local injection of 5% QX-314 into the central digit of the contralateral hindpaw ($n = 3$). Data from two different time periods (5–10 min and 25–30 min after QX-314 injection) are presented as a percentage of control responses before the injection. *B*, ACC responses to hindpaw stimulation were not affected by high-frequency stimulation (100 Hz, 1 s at the same intensity) of peripheral inputs at the central digit of the contralateral hindpaw ($n = 4$). Data from two different time periods (10–15 min and 25–30 min after high-frequency stimulation) are presented as a percentage of control responses before the injection. *C*, ACC responses to hindpaw stimulation were not affected by two trains of high-frequency stimulation (100 Hz, 1 s twice with a 20 s interval) of peripheral inputs at the central digit of the contralateral hindpaw ($n = 3$).

are needed to investigate these possibilities. We also provide evidence that brief silencing or stimulating of peripheral inputs from a single digit failed to reproduce the effects of amputation (i.e. increases in the ACC responses), indicating that amputation is a complex phenomenon. The abnormal nerve activities during and after amputation and permanent removal of peripheral inputs probably contribute to increases in the ACC responses.

Nociceptive responses in the ACC

Among many physiological functions of the ACC (D'Esposito *et al.* 1995; Botvinick *et al.* 1999), its role in pain and pain-related cognitive functions has been intensely investigated. The ACC has been suggested to contribute to the perception of pain, the learning process associated with the prediction/avoidance of noxious sensory stimuli as well as pathological phantom pain (see Devinsky *et al.* 1995 for a review). Electrophysiological recordings from both animals and humans demonstrate that neurons within the ACC respond to noxious stimuli (animals: Vogt *et al.* 1979; Devinsky *et al.* 1995; Sikes & Vogt, 1992; humans: Hutchison *et al.* 1999). In human studies with a combination of positron emission tomography (PET) and magnetic resonance imaging (MRI) it was found that the ACC showed significant responses to noxious heat stimuli (Talbot *et al.* 1991; Vogt *et al.* 1996; Craig *et al.* 1996). In the present study, we demonstrated that electrical foot shocks induce excitatory sensory responses from the ACC. Evoked responses had a long latency to onset, suggesting that sensory inputs relay on different nuclei along somatosensory pathways. Future studies taking advantage of *in vivo* preparations should identify individual nuclei involved in mediating evoked responses. It is worth pointing out that evoked responses required stimulation at high intensities, and stimulation at low intensities induced a lower or no response. This finding favours the idea that the ACC is more likely to be activated by noxious stimuli. However, the present findings do not exclude the possibility that the ACC neurons also respond to non-noxious stimuli as well as to other sensory information related to the environment that the animals are in the conscious state. Although there is compelling evidence for the involvement of the ACC in nociception and pain, one should keep in the mind that the participation of the ACC is also well documented in other types of cognitive function.

Long-lasting changes in the ACC after amputation *in vitro* and *in vivo*

Glutamate is a major fast excitatory transmitter within the ACC (Sah & Nicoll, 1991; Tanaka & North, 1994; Wei *et al.* 1999). The long-term plasticity of glutamatergic synapses is well documented in the hippocampus and includes long-term potentiation and long-term depression (LTD) (for reviews see Bliss & Collingridge, 1993; Bear & Malenka, 1994; Zhuo & Hawkins, 1995). Unlike the hippocampus, only a few studies have been performed in

the ACC slices *in vitro*. Sah & Nicoll (1991) reported that a brief tetanic stimulation of the afferent fibres (callosal inputs) induced synaptic potentiation lasting less than 1 h. We similarly found that strong tetanic stimulation (two trains at 100 Hz) did not induce long-term potentiation (Wei *et al.* 1999). Low-frequency stimulation (1 Hz for 15 min) induced synaptic depression lasting for at least 30 min after the stimulation (Wei *et al.* 1999). These results indicate that glutamatergic synapses in the ACC share some but not all of the synaptic plasticity of the hippocampus. More importantly, it is necessary to see whether a similar stimulation paradigm could induce long-term potentiation/depression *in vivo* and to explore whether synaptic responses undergo plastic changes under physiological and/or pathological conditions. In a recent report (Wei *et al.* 1999), we demonstrated that plastic changes occurred within the ACC after amputation, using *in vitro* cingulate slices and immunostaining techniques. Unlike in other areas of the brain such as the hippocampus and somatosensory cortex, LTD is the major form of synaptic plasticity in cingulate slices from adult rats. Furthermore, significant decreases or a complete loss of synaptic depression was found in slices collected from animals following digit amputation. Because many IEGs have been also expressed in a similar area within the cingulate cortex, we argue that digit amputation causes long-term plastic changes within the ACC. The results of the present study further support our hypothesis. We here provide *in vivo* electrophysiological evidence that sensory responses in the ACC to noxious stimuli are significantly enhanced after amputation. Interestingly, these increases were long-lasting (more than 2 h). Because amputation was performed at the other, normal hindpaw, it is most likely that enhancement was due to central plasticity occurring at the level of the spinal cord or higher structures.

We always feel that it is difficult to *directly* compare results from *in vitro* brain slices with those from *in vivo* animals. In a previous study using cortical slices, we showed that LTD induced by low-frequency repetitive stimulation was significantly decreased or diminished after amputation (Wei *et al.* 1999). It is possible that alterations in synaptic excitability (at presynaptic and/or postsynaptic sites) caused by amputation affect the induction of LTD. There are at least two important differences between slice and animal experiments. First, many inputs from other regions of the brain (e.g. serotonergic inputs from raphe nuclei to the ACC) are removed in slice preparations. Therefore, the slice preparation presents a rather simple version of actual brain circuitry. Second, unlike in *in vivo* experiments, we cannot compare responses/plasticity before amputation with those after amputation in slice experiments. If we assume that the loss of LTD in slices and increases in the ACC responses *in vivo* happen in the same population of excitatory synapses in the ACC (see the beginning of the Discussion), one possible mechanism is that loss of LTD (known as dis-depression) may favour the occurrence of enhancement upon the same input stimulation.

Both *in vitro* and *in vivo* data suggest that one probable site undergoing these changes is the ACC. Digit amputation caused significant, long-lasting increases in the ACC responses induced by local ACC stimulation, arguing that changes within the ACC may contribute to the enhancement of evoked excitatory synaptic responses to peripheral foot shocks. This argument is also supported by paired-pulse stimulation experiments. We showed that ACC focal stimulation and hindpaw stimulation caused a similar amount of depression of synaptic responses to subsequent hindpaw stimulation, suggesting that intra-ACC stimulation and peripheral hindpaw stimulation at least activate some common pathways. We would like to point out that the results of paired-pulse depression may be explained differently if the two inputs (i.e. hindpaw and local ACC inputs) share some common inhibitory interneurons. In this case, cross-paired-pulse depression between two sets of inputs might result from the activation of a common group of inhibitory neurons while non-overlapping populations of excitatory cells are activated by hindpaw or local ACC stimulation. Our *in vivo* recording technique cannot exclude this possibility, due to the polysynaptic nature of the responses we studied.

Our results do not exclude potential changes in other synaptic connections (such as the tonic level of activity or inhibition) as well as in other somatosensory nuclei including the spinal cord dorsal horn. In fact, hindpaw brushing induced significantly increased c-fos immunostaining in dorsal horn neurones after amputation of a digit from the other hindpaw (authors' unpublished observations), mirroring the increased expression observed in the ACC. Although the exact cellular mechanism is unclear, one possible explanation is that descending or local spinal modulatory pathways may be affected after amputation. The significance of the present study is that it provides the first evidence that sensory responses of the ACC to peripheral noxious shocks or local cortical stimulation can be enhanced for a long time after amputation. Future studies using different approaches including *in vivo* intracellular recording may help to identify the possible plastic changes in other central areas.

Functional implications

Evidence from animals and humans consistently demonstrates that the ACC plays an important role in nociception and pain. Lesion of the rat medial frontal cortex including the ACC significantly increased behavioural response latencies to noxious hot or cold stimuli (Pastoriza *et al.* 1996; Lee *et al.* 1999). Electrical or chemical stimulation delivered to the sites within the ACC facilitated the rat spinal nociceptive tail-flick reflex (Calejesan *et al.* 2000). The unpleasantness of pain is abolished in patients with frontal lobotomies or

cingulotomies (Foltz & White, 1962; Hurt & Ballantine, 1973; Yarnitsky *et al.* 1988). Studies using IEG-encoded protein immunostaining techniques and *in vitro* neuronal plasticity showed that neurons within the ACC could show plastic changes following amputation (Wei *et al.* 1999). Together with the present results, we suggest that long-lasting enhancement in the ACC after amputation may play an important role in abnormal nociceptive responses after amputation. Behavioural studies in amputated rats revealed hyperalgesia in response to noxious stimuli (authors' unpublished data). Although it is unlikely that enhanced sensory transmission within the ACC alone could explain behavioural hyperalgesia, long-lasting enhancement within the ACC could certainly contribute to various pain-related functional alterations after amputation. For example, synaptic potentiation may trigger the late-stage changes in neuronal structures that have been reported after amputation (see Introduction).

In the present study, we did not address potential changes in other cortical and subcortical areas such as the somatosensory cortex, thalamus and spinal cord. It is possible that similar plastic changes may occur in these areas as well (see Kaas *et al.* 1999). Understanding plastic changes within the areas involved in pain transmission and modulation after amputation could allow us to treat phantom pain in human amputees. The present study suggests that plastic changes occurring within the ACC after amputation may provide a possible synaptic mechanism for phantom pain. Because the ACC has been suggested to contribute to the emotional unpleasantness of pain in humans, it is conceivable that an enhanced excitability of neurones within the ACC may lead to a perception of pain triggered by a somatosensory input that is not normally painful and thus does not normally lead to ACC activation (Baron & Maier, 1995; Lorenz *et al.* 1998). A better understanding of the molecular mechanisms involved in synaptic changes in the ACC may eventually help us to control phantom pain in human amputees.

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