Research Paper

Biphasic modulation of behavioral nociceptive responses by morphine in adult mice after amputation

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Abstract: Amputation of a segment of the tail produced long-lasting changes in nociception and morphine-induced antinociception. Plastic changes in nociceptive transmission may occur at the spinal cord as well as supraspinal structures after tail amputation. Acute hyperalgesia is detected at the remaining part of the tail as well as hindpaw. Morphine induced facilitation of the hot-plate (HP) response at a low dose and a greater dose of morphine is required to produce complete inhibition of the HP response. Since these effects happen at five weeks after the surgery, tail amputation may serve as a mouse model for studying long-term plastic changes in central nervous system after amputation.

Key words: amputation; nociception; antinociception; facilitation; morphine; mice; phantom pain

Peripheral tissue and/or nerve injury often cause persistent nociception in animals and chronic pain in humans which last for an extended period of time. Peripheral tissue injury is associated with persistent nociception and chronic pain in humans. Both hyperalgesia (enhanced response to noxious stimuli and increase in intensity of pain) and allodynia (pain induced by non-noxious stimuli) are associated with persistent nociception/pain. Central long-term plasticity after injury is believed to contribute to the induction and maintenance of persistent pain. Such long-term plastic changes happen not only at the level of the spinal cord, but also in the supraspinal cortical areas such as the anterior cingulate cortex. Although peripheral increased sensitivity of primary afferent nociceptors (includes lowered thresholds and an increased responsive-
ness of the skin) are thought to be required for behavioral nociceptive responses after injury, long-term enhancement of central neuronal excitability plays an important role in enhanced long-term behavioral responses to injury\(^{[4-6]}\). Understanding molecular and cellular mechanisms for long-term plasticity changes are thought to be critical for designing effective treatment for chronic pain in patients\(^{[4]}\).

Genetic deletion of a selected protein in mice provides a powerful tool for investigating molecular mechanisms of various physiological functions of the central nervous system such as synaptic plasticity, learning and memory, motor functions, pain and analgesia\(^{[9-13]}\). For example, in mice with selective forebrain overexpression of NMDA NR2B receptors, persistent pain but not acute pain is selectively enhanced in transgenic mice\(^{[9]}\). By contrast, in double knockout mice of calcium-calmodulin stimulated AC1 and AC8, persistent pain is selectively reduced or abolished while acute pain is not affected\(^{[11]}\). These studies provide new directions for searching new generation of drugs to selectively target chronic pain while leaving acute pain intact, considering the ability to respond to acute noxious stimuli is the essential protective function for humans and animals in their living environment\(^{[4-6]}\).

In order to investigate molecular and cellular mechanisms underlying persistent pain, we have established tail or digit amputation model for investigating synaptic mechanisms for phantom pain\(^{[7,10,15]}\), a common clinical pain after amputation due to various diseases, accidence or war\(^{[16-24]}\). Considering the difference between rats and mice, we developed two different models for rats and mice respectively. For rats, we performed a single digit amputation. For mice, we performed the amputation of a distal tail. We found that nociceptive thermal and mechanical thresholds were decreased at 1–7 days after tail amputation in adult mice\(^{[15]}\). However, it is unclear if such hyperalgesia persists for a longer period of time and whether other changes such as persistent nociception and morphine-induced antinociception may also occur after tail amputation. In the present study, we studied acute nociception and morphine-induced antinociception at five weeks after amputation of a segment of the tail. We found that many properties of nociception/antinociception were significantly affected: hyperalgesia of the remaining part of the tail and normal hindpaw to noxious thermal (heat or cold) stimuli; decreases in morphine-induced antinociception in the hot-plate (HP) test; and facilitation of the HP test induced by morphine at a low dose. These findings suggest that plastic changes in central nociceptive processing happen after tail amputation and such changes are likely long-lasting.

### 1 MATERIAL AND METHODS

#### 1.1 Animals and surgical procedure

Adult male mice C57BL/6J weighing from 24–30 gram were used (Jackson). The room temperature was maintained at 20ºC. The experimental protocol was approved by the Animal Study Committee in Washington University and University of Toronto. Under halothane anesthesia, the distal 2.5 cm length of the tail was removed. Crazy glue was used to stop bleeding.

#### 1.2 Hot-plate test

The HP reflex was measured with a controlled metal plate (Columbia Instruments, Columbus, Ohio). The nociceptive response was licking, lifting hindpaw or jumping off the HP and the latency for response was recorded with a digital timer. Mice were removed from the HP immediately after the first response. The metal plate temperature was maintained at 50 or 55ºC. Two different cut-off times were used to prevent damage to the hindpaw skin, 30 s for HP at 55ºC and 60 s for HP at 50ºC. Mice were removed from the chamber if they did not respond within 30 or 60 s.

#### 1.3 Spinal nociceptive tail-flick reflex

The spinal nociceptive tail-flick (TF) reflex was evoked by noxious radiant heat focused on a circular hole (8 mm in diameter) the underside of the tail plate (Columbia Instruments, Columbus, Ohio). The latency to reflexive removal of the tail from the heat was measured by a digital photocell timer to the nearest 0.1 s. A cut-off latency of 12 s was used to avoid damage to the skin. The baseline threshold was the mean of 2 or 3 measurements.

#### 1.4 Cold-plate test

Ice in a plastic container (21 cm diameter × 21 cm height) was used as a cold-plate. The temperature of the ice surface was monitored with a digital thermometer and maintained at 0ºC. The time between the placement of a mouse on the cold-plate and the first jumping response was measured with a digital timer. Mice were removed from the plate after the first jumping response or a cut-off time (60 s) had passed\(^{[25]}\).

#### 1.5 Drug preparation and administration

Morphine sulfate was freshly dissolved in sterile saline (0.9% in NaCl). Different doses of morphine were injected intraperitoneally (i.p.) at a cumulative fashion with a 30 min interval. Nociceptive responses were evaluated every 10 min and measurements at 30 min after each injection were used for evaluating the ED50s of morphine.

The intrathecal (i.th.) injection was carried out as pre-
viously described[26]. Mice were anesthetized with halothane (2%) during the injection. After the injection, it took 2~3 min for mice to recover from halothane anesthesia. The volume of the injection was 5 µl. Drugs were always prepared freshly on the day of experiments and dissolved in sterile saline (0.9% NaCl).

1.6 Data and analysis. Data are presented as their mean value±one standard error of the mean (S.E.M.). Hyperalgesia is also presented as a percentage of the control. For antinociceptive effect, data is also presented as maximum possible effect (MPE). MPE = (observed threshold-baseline threshold)/(cut-off value-baseline threshold) ×100. Negative MPE values indicate that the treatment produces facilitatory effects. Statistical comparisons were made with the use of one-way or two-way analyses of variance (ANOVAs; Newman-Keul’s test for post-hoc comparison). Student’s t test was applied for comparisons between groups. In all cases, P<0.05 was considered significant.

2 RESULTS

2.1 Long-term hyperalgesia after amputation in adult mice

To study changes of behavioral responses to noxious sensory stimuli after amputation, we employed several behavioral tests: (1) hot-plate test: a reflexive response requiring both spinal and supraspinal structures; (2) the spinal nociceptive tail-flick reflex: a spinal reflex does not require the involvement of supraspinal structures, however, it may be influenced by descending modulation systems. Injury caused long-term hyperalgesia in adult animals. Supporting our previous reports of hyperalgesia after amputation[15], we found that at five weeks after amputation of a segment of the tail, hyperalgesia to noxious heat stimuli was observed at the remaining part of the tail. Hyperalgesia of the hindpaw to noxious heat (55ºC) stimuli was observed in amputated mice. The mean HP latency was 11.3 ± 0.9 s (n=20) in amputated mice and 14.6 ± 0.7 s in normal mice (n=36) (Fig. 1). There is significant difference in HP latency between normal and amputated mice (t (54)=2.83; P<0.005). In some animals, HP responses at 50ºC were also tested. HP latency at 50ºC was similar between normal mice (n=7; 25.5 ± 2.4 s) and amputated mice (n=10; 24.0 ± 1.9 s) (t (15)=0.542; P=0.297). These findings suggest that thermal hyperalgesia is temperature-dependent. One possible explanation for different HP results at 50ºC vs 55ºC is that amputation-related enhancement becomes more significant at a higher temperature. Therefore, changes in the HP responses latency can be observed. We do not rule out possible subthreshold changes at 50ºC, it may be detected with more sensitive tests.

Hyperalgesia is not limited to the hindpaw. TF latency in the remaining part of the tail was also decreased after tail amputation. In a total of thirteen amputated mice, the mean TF latency was 5.2 ± 0.3 s (n=13). The mean TF latency in ten normal mice was 6.5 ± 0.2 s (Fig. 1). TF latency in amputated mice is significantly shorter than that of normal mice (t (21)=3.31; P<0.005). In six mice, TF latencies were measured before and after tail amputation. TF latency was significantly decreased to 77.8 ± 5.6% of control at five weeks after tail amputation (6.4 ± 0.1 s pre vs 5.0 ± 0.3 s post; t (5)=3.77; P<0.01).

2.2 Facilitation of the hot-plate responses after a low dose of systemic morphine following amputation

One important clinical problem with injury is that morphine, a potent pain killer, often loses its effects in animals or humans with chronic pain[27]. To determine if antinociception induced by morphine may also be affected...
in amputated mice, we performed experiments to determine the dose-dependent antinociception produced by morphine in the HP test and the TF reflex. First, in the HP (50°C) test, we tested the effects of morphine at different doses. Interestingly, at a low dose of morphine (0.1mg/kg), morphine injected i.p. produced a significant facilitation of HP response in amputated mice \((n=10); 27.0 \pm 0.9\) s pre vs 17.4 ± 2.4 s post; 30.0 ± 8.5% change). By contrast, morphine (0.1 mg/kg) did not induce any facilitation of the HP response in control mice \((n=7)\) (Fig. 2). At a high dose (1 mg/kg), morphine did not produce any significant change in HP latencies (Fig. 2). At doses of 5 and 10 mg/kg, morphine produced significantly increases in HP latencies \((n=7\) mice for each dose). As compared with control mice, morphine-produced inhibitory effects were significantly reduced. In amputated mice, the ED50 (dose to produce

Fig. 2. Facilitation of the hot-plate responses by a low dose of morphine and reduced morphine analgesia in amputated mice. A: The effects of systemic morphine at a low dose (0.1 mg/kg, i.p.) on the hot-plate response latencies in normal (open bar) and amputated (filled bar) mice. B: Dose-dependent effects of systemic morphine on the hot-plate responses latencies in normal and amputated mice. Data are presented as mean±SEM. *\(P<0.05\) as compared with control group.

Fig. 3. Effects of systemic morphine on the spinal nociceptive tail-flick in normal and amputated mice. A: The effects of systemic morphine at a low dose (0.1 mg/kg, i.p.) on the tail-flick response latencies in normal (open bar) and amputated (filled bar) mice. B: Dose dependent effects of systemic morphine on the tail-flick responses latencies in normal and amputated mice. Data are presented as mean ± SEM. *\(P<0.05\) as compared with control group.
inhibition to 50% of maximum) for morphine was significantly greater than that in normal mice \((F(1,46)=14.505; P<0.001)\) (see Fig. 2).

### 2.3 No facilitation of spinal nociceptive tail-flick reflex

Since the hot-plate test requires the involvement of both spinal and supraspinal structures, the low-dose morphine may affect spinal and/or supraspinal sensory process to cause facilitatory effects. To determine possible spinal effects of low-dose morphine, we used the spinal nociceptive tail-flick reflex. Different from the HP test, morphine \((0.1\, \text{mg/kg})\) did not produce any facilitation either in the tail-flick reflex \((n=7\, \text{mice for each group})\), indicating that the morphine-produced facilitatory effects may be supraspinal (Fig. 3). Furthermore, morphine at a high dose of \(10\, \text{mg/kg}\) produced similar antinociceptive effects in the tail-flick reflex in amputated and control mice \((n=7\, \text{mice for each group})\). However, at a dose of \(5\, \text{mg/kg}\), morphine produced significantly less inhibition in amputated mice \((n=7\, \text{mice})\) as compared with that in control mice \((n=7\, \text{mice}; \text{Fig. 3})\).

### 2.4 Spinal injection of morphine after amputation

Systemic morphine may act on opioid receptors at different locations, to further determine the contribution of central loci of opioids, we administered local injection of morphine into the spinal cord at different doses. As shown in Fig. 4, we did not find any facilitation of the HP responses after morphine injection. Intrathecal administration of morphine at different doses \((0.5\, \text{and} \, 12.5\, \text{µg})\) produced dose-dependent inhibition of the hot-plate tests in amputated \((n=5\, \text{mice})\) and control \((n=6\, \text{mice})\). No significant difference was found between the two groups of mice. Similarly, intrathecal administration of morphine produced similar dose-dependent inhibition of the tail-flick reflex, indicating that spinal antinociceptive mechanisms for morphine were not significantly altered by digit amputation. These findings further support the hypothesis that the action of morphine-produced facilitation is likely to be supraspinal.

### 2.5 Cold-hyperalgesia after injury

It is known that noxious cold also induce escape behaviors in animals and pain in humans. As previously reported\cite{15}, hyperalgesia of the hindpaw to noxious cold \((0^\circ\text{C})\) stimuli were observed in amputated mice. In thirteen mice tested, the mean cold-plate latency was \(11.9 \pm 2.4\, \text{s}\) at five weeks after tail amputation. However, in normal mice, the mean cold-plate latency was \(45.4 \pm 4.4\, \text{s}\) \((n=10)\) (Fig. 5). Cold-plate latency after tail amputation is significantly shorter than that in normal mice \((t(21)=7.11; P<0.0001)\). In six mice, cold-plate latencies before and after amputation were measured. The mean cold-plate latency was \(49.2 \pm 6.8\, \text{s}\)
before amputation and decreased to 32.4 ± 9.1% of control after tail amputation (13.2±2.7 s; t (5)=4.49; P<0.005 compared with cold-plate latency before amputation). Systemic administration of morphine produced dose-dependent inhibition of the cold-plate responses in amputated mice (n=7 mice). Unlike the hot-plate test, no facilitation was observed. Furthermore, as compared with the control mice, the antinociceptive effects in the cold-plate tests were not significantly affected in amputated mice (Fig. 5).

To examine possible contribution of spinal morphine produced antinociception, we also performed intrathecal administration of morphine. To our surprise, morphine at different doses did not produce any antinociceptive effects in amputated mice (n=6 mice). By contrast, intrathecal administration of morphine produced dose-dependent inhibition in the cold-plate responses of the control mice (n=6 mice; Fig. 6).

### 3 DISCUSSION

The results of the present study indicate that nociception as well as antinociception (by morphine) are significantly affected in amputated mice. Such alteration is long-lasting (>five weeks). Long-lasting hyperalgesia is found not only in the remaining part of the tail, but also in normal hindpaw, suggesting that central sensitization at the spinal cord and/or supraspinal structures contribute to wide-spread hyperalgesia. These findings are consistent with recent synaptic studies that long-term plastic changes happen in pain-related cortical regions such as the anterior cingulate cortex (ACC) and insulate cortex[7,9]. It is likely that enhanced neuronal excitibility in pain-related central nuclei contribute to the changes in morphine analgesia, in addition to possible peripheral and spinal plastic changes after the injury. Alteration in morphine produced antinocicep-

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**Fig. 5.** Effects of systemic morphine on the cold-plate responses in normal and amputated mice. **A:** The effects of systemic morphine at a low dose (0.1 mg/kg, i.p.) on the cold-plate response latencies in normal (open bar) and amputated (filled bar) mice; **B:** Dose-dependent effects of systemic morphine on the cold-plate responses latencies in normal and amputated mice. Data are presented as mean±SEM. *P<0.05 as compared with control group.

**Fig. 6.** Effects of intrathecal administration of morphine on the cold-plate responses in normal and amputated mice. Effects of intrathecal administration of morphine at two different doses on the cold-plate responses latencies in control (open bar) and amputated (filled bar) mice. Data are presented as mean ± SEM. *P<0.05 as compared with control group.
tion after amputation in mice are novel. Although the investigation of possible synaptic and molecular mechanisms for behavioral facilitation and morphine-produced antinociception are not the focus of the current study, our studies provide a mouse model for future studies of molecular mechanisms for morphine-produced facilitation and antinociception.

Results from acute nociception and morphine-induced antinociception suggest that nociceptive transmission in the central nervous system is significantly enhanced after tail amputation. Since spinal mediated TF reflex as well as supraspinal mediated HP response both were facilitated after tail amputation\cite{15}, the enhancement of nociceptive sensory transmission are likely happening at the spinal cord as well as at the supraspinal structures. In addition to well-documented changes in the spinal cord dorsal horn (see Zhuo, 2000; Willis, 2002), our recent animal studies with distal tail or a single digit amputation models reveal that amputation caused rapid expression of different immediate early genes in the cortex, including the ACC and hippocampus \cite{7,9,15}. Interestingly, changes in synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), have also been reported in both slices of amputated animals and whole animals\cite{7-10}. These recent findings provide strong physiological evidence that synaptic connections in the brain can be altered after amputation. While it is too early to speculate on the behavioral correlates of these synaptic changes, we did observe long-term changes in behavioral responses to noxious stimuli in the present study. Altered glutamate-mediated synaptic transmission in the ACC and possible changes in signaling pathways downstream from opioid receptors may contribute to changes or loss of morphine-produced nociception after amputation.

It is well documented that spinal nociception is under biphasic modulation, facilitatory and inhibitory modulation \cite{28-31}. Activation of neurons in the ACC or RVM at certain intensities produces facilitation of spinal nociceptive transmission and behavioral responses to noxious stimuli \cite{28-31,33}. It has been demonstrated that tonic descending facilitation from the RVM mediates opioid-induced abnormal pain and antinociceptive tolerance in animals\cite{34}. From clinic observations, it has been reported that systemic morphine induced hyperalgesia in patients with cancer pain\cite{35,36}. In the present study, we found that at a low dose (0.1 mg/kg), morphine induced facilitation of the HP response in amputated but not in normal mice. By contrast, no facilitation was observed in the TF reflex in amputated mice. The TF reflex is a spinal mediated nociceptive reflex whereas the HP response requires the involvement of supraspinal structures. Two possible mechanisms may contribute to the facilitation induced by morphine in amputated mice: enhancement of nociceptive transmission in the supraspinal sensory pathway which is critical for the HP response or selective facilitation of peripheral inputs from the hindpaw to dorsal horn neurons of the spinal cord by activation of descending facilitatory system from the brainstem. Future studies are needed to investigate if descending facilitatory influences from the rostroventral medulla (RVM) are involved in this effect\cite{28-32}.

From a clinical perspective, our studies provide a possible cellular model for studying long-term changes in nociception and morphine-induced antinociception. Long-lasting plastic changes may happen in the spinal cord as well as supraspinal structures such as the somatosensory cortex. Understanding mechanisms underlying these changes may help us to gain a better knowledge of pathophysiological changes in patients with amputation, in particular, phantom pain. The mouse tail amputation could serve as a animal model for exploring molecular and cellular mechanisms of phantom pain as well as changes in morphine-produced analgesia in chronic pain.

Acknowledgment:
We would like to thank Kalana Sheneice Thomas for technical help.

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