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Plenary Article

Exacerbation of tonic but not phasic pain by entorhinal cortex lesions

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HIGHLIGHTS

- We performed medial (MEC), lateral (LEC) or sham entorhinal cortex lesions in rats.
- Neither MEC nor LEC lesions affected the hot plate test.
- Neither MEC nor LEC lesions affected the first phase of formalin test.
- MEC and LEC lesions increased paw licking in the second phase of formalin test.

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ABSTRACT

The hippocampus is actively involved in pain modulation. Previous studies have shown that inhibition, resection or pharmacological interference of the hippocampus or its subcortical afferent sources such as the medial septum and amygdala produce anti-nociceptive effects. But how the cortical connections of the hippocampus modulate pain remains unexplored. The entorhinal cortex (EC) constitutes the major gateway between the hippocampus and the neocortex. In the present study, rats with medial (MEC), lateral (LEC) or sham EC lesions and received the hot plate and the intra-plantar formalin injection tests. Neither MEC nor LEC lesions affected the hot plate test and the first phase of the formalin test. In contrast, paw licking responses in the second phase of the formalin test significantly increased with both MEC and LEC lesions. These results suggested that that the hippocampal–cortical interactions channeled by the EC were involved in tonic but not phasic pain conditions, and that cortical and sub-cortical connections of the hippocampus played independent roles in pain modulation.

1. Introduction

The hippocampus actively participates in pain processing and modulation. Hippocampal neurons respond to noxious stimuli [1,2], whereas electrical stimulation of the hippocampal formation evokes painful sensations in humans [3]. Persistent pain in adult [4–6] and neonatal rodents [7–9] changes hippocampal morphology, electrophysiology and function, and affects its neurogenesis. Inhibition [10], resection [11] or pharmacological interference [12–16] of the hippocampus modulates formalin-induced pain behaviors.

Evidence suggests that the hippocampus plays distinct roles under different pain conditions. For example, hippocampal inhibition attenuates tonic pain induced by formalin injection [10,12,13], but no differences of phasic pain in the hot plate or tail flick tests are detected with hippocampal lesions [17]. The underlying mechanisms remain unclear, partly because of its complicated anatomy. Medial septum, amygdala, thalamus and hypothalamus send subcortical afferents to the hippocampus whereas its cortical connections almost exclusively pass through the entorhinal cortex (EC). Cortical information enters the hippocampus through layers II/III of the EC, and after processing, returns to these regions through EC deep layers. The EC can be further subdivided, on both anatomical and functional basis, into medial entorhinal cortex (MEC) and the lateral entorhinal cortex (LEC), which process spatial and non-spatial information, respectively [18–20].

The pain-modulatory effects of subcortical afferents of the hippocampus have been demonstrated by studies showing that inhibition, resection or stimulation of the medial septum [21,22], amygdala [23–25] and hypothalamus [26,27] alter pain behaviors. However, whether and how cortical connections of the hippocampus modulate pain remains unexplored. The present study aims to

Abbreviations: EC, entorhinal cortex; MEC, medial entorhinal cortex; LEC, lateral entorhinal cortex.

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examine how EC lesions, which disrupt the hippocampal–cortical gateway, affect phasic and tonic pain conditions.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats weighing 250–300 g at the beginning of the experiment were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. Rats were housed 4–6 per cage in a temperature and light-controlled room under a 12:12 h light:dark cycle with water and food provided *ad lib*. The animals were handled and habituated 7–10 days before any experiments. All animal experimental procedures were conducted in accordance with the guidelines of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of the University. The

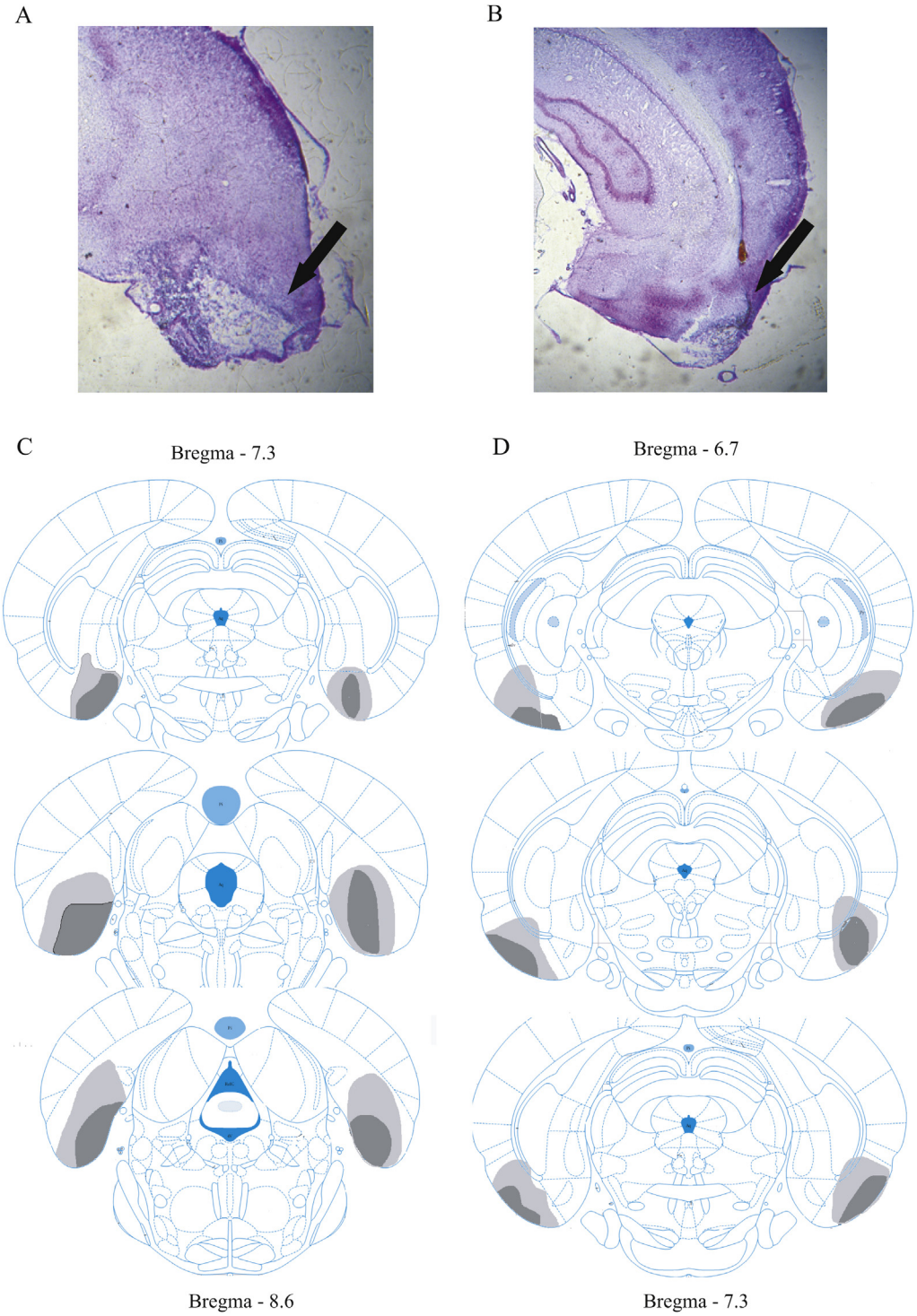

Fig. 1. Histology of MEC and LEC lesions. Arrows indicated sample MEC (A) and LEC (B) lesions in Nissl-stained coronal sections. Schematic representation of the maximum (grey) and minimum (dark) extents of lesions were shown in (C) and (D).

behavioral experimenters were kept blind from the groupings of the rats.

2.2. Entorhinal cortex lesions

Bilateral LEC ($n = 14$) and MEC ($n = 13$) lesions were performed under sodium pentobarbital anesthesia. A 1.0 mA current was passed through an insulated stainless steel electrode for 20 s at the following stereotaxic sites as previously described [20]: AP −7.3 mm, DL ± 6.0 mm, and AP −6.6 mm, DL ± 6.2 mm for LEC, and AP −8.3 mm, DL ± 4.8 mm, and AP −8.8 mm, DL ± 4.7 mm for MEC. The electrode was lowered to the bottom of the skull and then lifted 1 mm. Sham lesions followed the same procedure except that no electric currents were passed. 5 rats received sham LEC lesions and 6 sham MEC lesions. All rats were given 7 days to recover before the hot plate re-test and the formalin test.

2.3. Hot plate test

The hot plate test was taken as a phasic pain test. The temperature of the hot plate was set at 52 °C. Paw licking latencies were tested three times 1 week before and after lesions. Thirty seconds were taken as the cut-off time to avoid plantar injuries.

2.4. Formalin test

After two days' habituation to a 30 × 30 × 30 cm Plexiglas chamber, each rat was administered a 100 μl injection of 5% formalin solution into the plantar surface of the left hind paw. After injection, the rat was placed into the chamber for behavioral testing lasting 60 min. The amount of time the animal spent with the injected paw down, lifted or licked was recorded every 5 min. A weighted pain score for each animal was calculated using the following formula as previously described [28,29]: pain score = [time spent with injected paw elevated + 2 × (time spent licking injected paw)]/(total time). Data were also analyzed in the pooled manner with 0–10 min representing phase 1 of the formalin test and 10–60 min representing phase 2.

2.5. Histology

After all experiments, rats were deeply anesthetized and perfused with 4% paraformaldehyde in phosphate buffer. The brains were extracted and stored for 24 h in a paraformaldehyde solution. Thirty micrometer sections were sliced coronally using a cryostat microtome through the full EC and mounted on gelatin coated glass slides. After air drying for 3 days, the sections were Nissl stained with cresyl violet for microscopic visualization of the lesion.

2.6. Statistics

The effects of lesions were analyzed by one-way ANOVA with Tukey post hoc tests. All results are presented as means ± S.E.M. In all statistical comparisons, p values < 0.05 were considered to be significant.

3. Results

3.1. Histology

Fig. 1 provided a schematic representation of MEC and LEC lesions. From normal rat brains, we calculated the MEC and LEC lesion extents. Eleven rats sustained an average of 77% (65–85%) bilateral tissue loss in the MEC lesion group (Fig. 1A) and eleven rats in the LEC lesion group showed 72% (40–85%) tissue loss (Fig. 1B). The minimum and maximum lesion extents were represented in dark and grey, respectively (Fig. 1C and D). For the remaining rats in these groups, three showed very limited unilateral MEC or LEC lesions, and one showed lesions of unilateral retrosplenial dysgranular cortex and parasubiculum instead of EC. In addition, one rat with sham lesion and one with LEC lesion experienced severe hemorrhage during surgery. Data from these subjects were omitted from analysis.

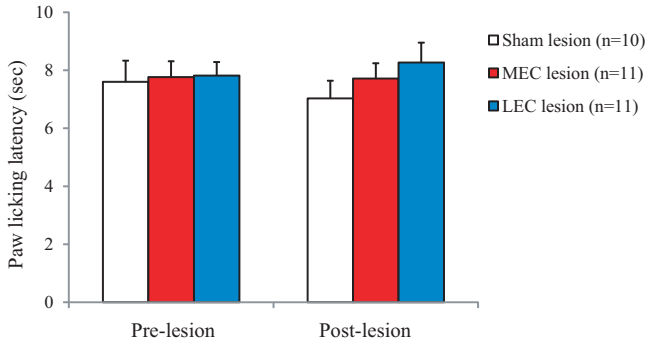

Fig. 2. Effects of MEC and LEC lesions on the hot plate test. Neither MEC nor LEC lesions affected paw licking latencies in the hot plate test.

Electrical lesions affect fibers of passage in addition to neurons. But this method is commonly used in EC studies (e.g. [20]). Previous studies have shown that except for some direct perirhinal inputs which do not pass through the EC, the vast majority of the cortical afferents to the hippocampus show synaptic relays in the EC [30,31]. So the behavioral effects in the present study would be more likely to be the consequence of EC lesions rather than damages of the passing fibers.

3.2. Hot plate test

In both pre- and post-lesion phases, neither LEC nor MEC lesioned rats showed differences from sham lesion controls in the hot plate test, indicating limited role of the EC in phasic pain (pre-lesion phase: $F_{2,32} = 0.035$, $p = 0.966$; Post-lesion phase: $F_{2,32} = 1.014$, $p = 0.375$, one-way ANOVA with Tukey post hoc tests, Fig. 2).

3.3. Formalin test

Intra-plantar formalin injection resulted in typical biphasic patterns of lifting and licking of the injected paw (Fig. 3A–C). The first phase of intense nociceptive behavior was observed in the first 5 min period following formalin injection. Thereafter, licking decreased in the second 5 min period, rose after about 10 min and lasted 50 min. Compared with the sham lesion group, both MEC and LEC lesion groups had higher pain scores in the second but not the first phase of the formalin test (phase 1: $F_{2,32} = 0.131$, $p = 0.878$; phase 2: $F_{2,32} = 7.599$, $p = 0.002$, one-way ANOVA with Tukey post hoc tests, Fig. 3A and D). A detailed examination of the behavioral responses indicated that the higher pain scores of the lesion groups mainly stemmed from increased paw licking behaviors (phase 1: $F_{2,32} = 0.397$, $p = 0.676$; phase 2: $F_{2,32} = 9.531$, $p = 0.001$, one-way ANOVA with Tukey post hoc tests, Fig. 3B and E) whereas the paw lifting behaviors were not different across groups (phase 1: $F_{2,32} = 0.619$, $p = 0.546$; phase 2: $F_{2,32} = 0.838$, $p = 0.443$, one-way ANOVA with Tukey post hoc tests, Fig. 3C and F).

4. Discussion

The hot plate test and the formalin test are routinely used pain tests in rodents with distinct mechanisms. The formalin test

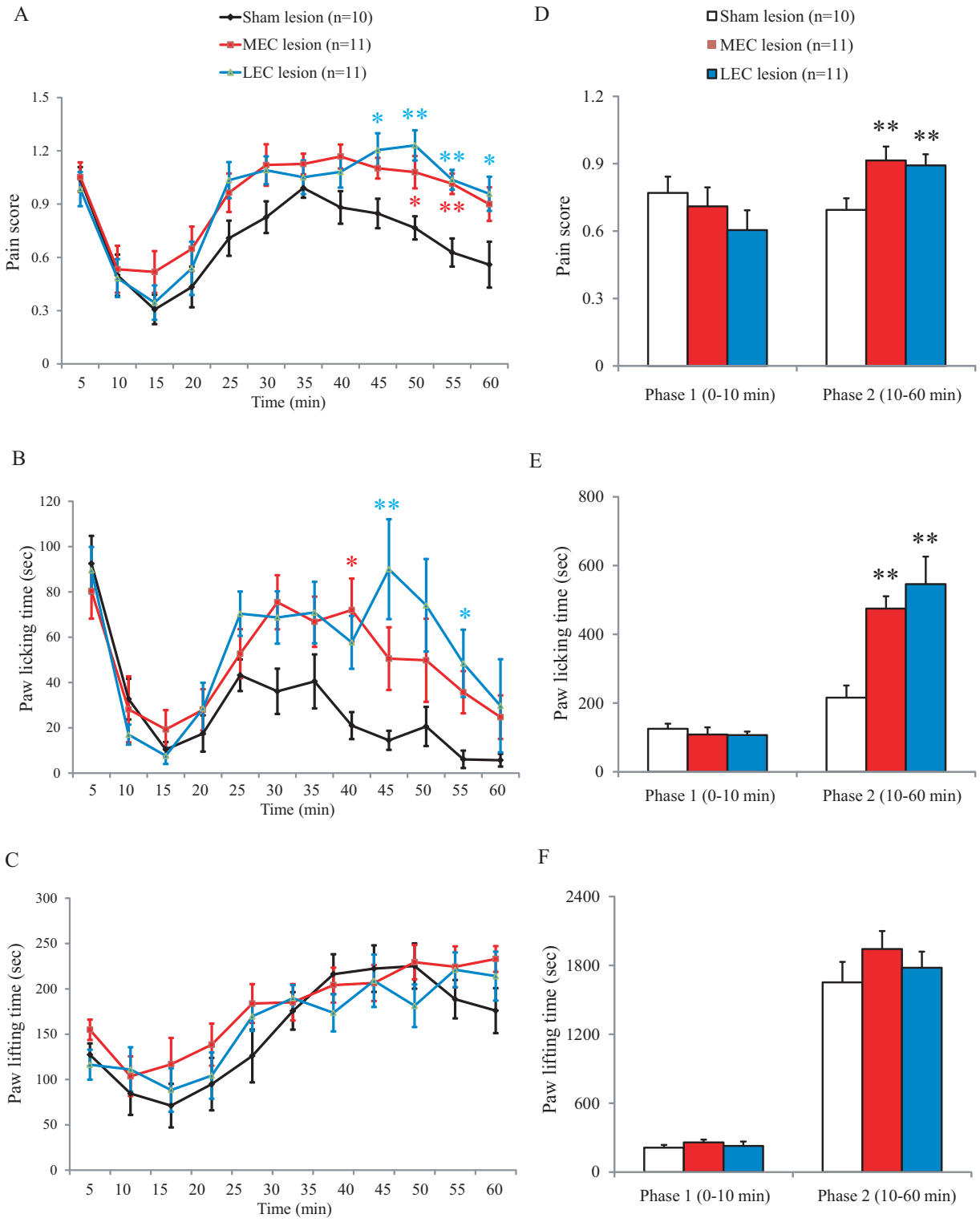

Fig. 3. Effects of MEC and LEC lesions on the formalin test. Pain score (A), paw licking time (B) and paw lifting time (C) curves of the formalin test revealed typical two-phase nociceptive behaviors induced by formalin injection. The pooled phase data were shown in (D), (E) and (F). MEC and LEC lesions significantly increased pain scores and paw licking time in the second phase of the formalin test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the sham lesion group.

consists of two distinct phases. The first 5 min phase is generally considered to be a peripheral response reflecting nociceptor activation, while the second, much longer phase results from the development of inflammation and central sensitization of both spinal and supraspinal levels [28]. In addition, two types of nociceptive behaviors observed in this test represent different mechanisms. Paw lifting and flinching represent spinally mediated responses whereas paw licking behaviors are associated with forebrain activities [28,32].

Results from the present study excluded a crucial role of the EC in pure peripheral mechanisms of pain behaviors, including the paw lifting responses of the whole formalin test and the paw licking

responses of its first phase. This was consistent with the absence of direct connections between peripheral nociceptors and the EC. In contrast, significantly exacerbated paw licking responses were observed in the second phase. Previous studies have shown that nociceptive responses of the formalin test are correlated with activity changes of both the hippocampus and the forebrain [2–5,21,32]. The prelimbic, infralimbic and anterior cingulate cortices have extensive connections with the hippocampus channeled through the EC and could contribute to EC lesions' effects on the second phase of the formalin test. However, the present study is not sufficient to specifically elucidate whether the effects stem from EC afferents to the hippocampus or hippocampal efferents to the EC or both.

In contrast to the formalin test which represents tonic pain, the hot plate test is a classical phasic pain test. The rat must execute hindpaw licking to escape from the stimulation when the level of heat exceeds the threshold of nociceptors. This response requires sensory-motor integration at the supraspinal level, and in particular, the medial frontal cortex [33]. Importantly, hippocampal lesions in adult rats did not affect the hot plate test [17], excluding the involvement of hippocampal–neocortical interactions. This was confirmed by our data that EC lesions did not affect this test.

If we broaden our viewpoint from the EC to the limbic system, most experiments prove that lesioning or inactivating the hippocampus results in analgesia [10–16]. Given that the EC provides the predominant excitatory drive to the hippocampus, EC-lesion-induced pro- but not anti-nociceptive effects in the formalin test were unexpected. In particular, inhibition or lesioning of the medial septum [21,22] and the amygdala [23–25], which sends strong sub-cortical afferents to the hippocampus, yields anti-nociceptive effects similar to that of the hippocampus itself. That means, subcortical and cortical connections of the hippocampus play independent roles in pain modulation. The pro-nociceptive role of EC lesions mimics disrupted inhibitory response control revealed by a recent study with disconnection of the hippocampal-prefrontal cortical circuit [34]. But there is a significant difference between these conditions. In studies on executive function, hippocampal lesions [35], ventral prefrontal cortical lesions [36] and hippocampal-prefrontal disconnection [34] produced consistent behavioral disinhibition. However, these manipulations yielded distinct effects in tonic pain. Considering the significant contribution of the hippocampal–neocortical interaction in various physiological processes, EC could be an important neural substrate for the cognitive and emotional modulation of pain in rodents or humans. Additionally, EC-mediated mechanisms could contribute to the cognitive and emotional changes in chronic pain states, where both hippocampal and cortical changes have been reported [2,4–6,37]. The distinction between MEC and LEC had been revealed in declarative learning and memory [18–20]. The present study did not involve specific cognitive demands, and no differences between LEC and MEC lesions were detected. Both contextual cues [38] and novel objects [39] have been reported to affect pain perception. Whether their modulation over pain is differentially mediated be MEC and LEC respectively is an interesting question for future research.

Conflict of interest

The authors claim no competing interests.

Contribution

Y.Z., F.F.L. and M.Y. performed the experiment; F.Y.L., Y.W. and M.Y. designed the experiment. All authors wrote the manuscript.

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