Interactions of CaMKII with dopamine D2 receptors: roles in levodopa-induced dyskinesia in 6-hydroxydopamine lesioned Parkinson’s rats

SuFang Zhang1, ChengLong Xie1, Qiang Wang2 & ZhenGuo Liu1

1Department of Neurology, Xinhua Hospital affiliated to the Medical School of Shanghai Jiaotong University, Shanghai, China, 2Department of Anesthesiology, School of Medicine, University of Missouri-Kansas City, Kansas City, MO 64108, USA.

Ca2+/calmodulin-dependent protein kinase II (CaMKII) is ubiquitously expressed in the central nervous system and is particularly enriched at excitatory synapses. This kinase is actively involved in the regulation of essential neuronal activities, including neurotransmitter synthesis and release, postsynaptic receptor signaling, and long-term synaptic plasticity. Available data indicate that CaMKII is linked to the pathogenesis and symptomatology of a variety of mental and neurological illnesses, including learning disorder, cognitive impairment, schizophrenia, ischemic, Alzheimer disease, epileptic seizures and PD. Recent evidence suggests a possible role of CaMKII in LID. Pharmacological inhibition of CaMKII with a selective inhibitor KN93 ameliorated dyskinesia in a rat PD model. However, how CaMKII in striatal neurons responds to and mediates LID is unclear. CaMKIIz has been demonstrated to interact with a number of synaptic receptors, including ionotropic and metabotropic glutamate receptors and dopamine D3 receptors, and by directly interacting with these receptors CaMKIIZ vigorously regulates their subcellular distribution and function. It is unknown at present whether CaMKII interacts with D2Rs and if so whether CaMKII-D2R interactions are sensitive to levodopa-induced dyskinesia.

Parkinson’s disease (PD) is primarily a result of selective death of dopaminergic neurons in the substantia nigra and consequential loss of dopamine innervations in the striatum. Dopamine replacement therapy with levodopa is so far the most effective therapy. However, chronic levodopa treatment causes abnormal involuntary movements (AIM) known as levodopa-induced dyskinesia (LID), for which the underlying molecular mechanisms are far from clear. Aberrant dopaminergic transmission is considered as a central element in the pathogenesis of LID. In the striatum, both dopamine D1 receptors and dopamine D2 receptors (D2R) are highly expressed. D1 receptors are known to be predominantly expressed in the direct pathway, i.e., striatonigral projection neurons, as opposed to the fact that D2Rs are expressed in the indirect pathway, striatopallidal neurons. While D1 receptors have been extensively studied for their participations in LID, the role of D2Rs in LID is poorly understood.

Ca2+/calmodulin-dependent protein kinase II (CaMKII) is ubiquitously expressed in the central nervous system and is particularly enriched at excitatory synapses. This kinase is actively involved in the regulation of essential neuronal activities, including neurotransmitter synthesis and release, postsynaptic receptor signaling, and long-term synaptic plasticity. Available data indicate that CaMKII is linked to the pathogenesis and symptomatology of a variety of mental and neurological illnesses, including learning disorder, cognitive impairment, schizophrenia, ischemic, Alzheimer disease, epileptic seizures and PD. Recent evidence suggests a possible role of CaMKII in LID. Pharmacological inhibition of CaMKII with a selective inhibitor KN93 ameliorated dyskinesia in a rat PD model. However, how CaMKII in striatal neurons responds to and mediates LID is unclear. CaMKIIz has been demonstrated to interact with a number of synaptic receptors, including ionotropic and metabotropic glutamate receptors and dopamine D3 receptors, and by directly interacting with these receptors CaMKIIZ vigorously regulates their subcellular distribution and function. It is unknown at present whether CaMKII interacts with D2Rs and if so whether CaMKII-D2R interactions are sensitive to levodopa and contribute to the development of LID.
In this study, we thus examined the relationship between CaMKII and D2Rs in vitro and in vivo. We first investigated whether recombinant CaMKIIξ proteins bind to purified D2Rs in vitro and whether their binding is direct. We then mapped a confined binding sequence from a CaMKIIξ binding region on the intracellular domain of D2Rs. To determine if native CaMKIIξ and D2Rs interact with each other, we carried out communoprecipitation with adult rat striatal lysates. Finally, we tested CaMKIIξ-D2R interactions in a rat PD model. We analyzed the changes in CaMKIIξ-D2R interactions in striatal neurons in response to chronic levodopa therapy. We also carried out behavioral experiments to determine the role of CaMKIIξ-D2R interactions in levodopa-induced dyskinetic behaviors.

Results

CaMKIIξ binds to D2Rs. D2Rs contain a characteristically long IL3, usually providing a site for protein-protein interactions. To determine whether CaMKIIξ interacts with IL3, we prepared a GST-fusion protein containing IL3 (GST-D2R-IL3) based on the long form of D2Rs as this form is preferentially involved in postsynaptic D2R signaling. We then used GST-D2R-IL3 as immobilized baits to precipitate endogenous CaMKIIξ from rat striatal lysates in pull-down assays. As shown in Fig. 1A, GST-D2R-IL3 precipitated CaMKIIξ, while GST alone did not. Two other GST-fusion proteins containing IL2 (GST-D2R-IL2) and CT (GST-D2R-CT) were also tested together. They showed no ability to precipitate CaMKIIξ (Fig. 1A). These results demonstrate the existence of interactions between CaMKIIξ and D2R-IL3. To determine whether CaMKIIξ directly interacts with D2Rs, we carried out in vitro binding assays with purified proteins. Purified CaMKIIξ was found to bind to the immobilized GST-D2R-IL3 (Fig. 1B). CaMKIIξ did not bind to GST alone, GST-D2R-IL2, and GST-D2R-CT (Fig. 1B). Thus, CaMKIIξ has the ability to directly bind to D2Rs through the D2R IL3 domain.

CaMKIIξ binds to the distal region of D2R IL3. To identify an accurate CaMKIIξ binding site in D2R IL3, we synthesized an N-terminal region of IL3, GST-D2R-IL3(K211-M270), and a C-terminal region of IL3, GST-D2R-IL3(K211-M374). GST-D2R-IL3(K211-M270) did not precipitate CaMKIIξ in binding assays (Fig. 2A). In contrast, GST-D2R-IL3(G242-Q374) precipitated the kinase to an extent similar to full length IL3. Thus, the C-terminal region of D2R IL3 contains a site that harbors CaMKIIξ binding. To further narrow down the sequence required for the CaMKIIξ binding, we synthesized a series of truncated IL3 fragments. A short distal IL3 fragment, GST-D2R-IL3(Q345-Q374), strongly precipitated CaMKIIξ (Fig. 2B). Other two fragments proximal to Q345-Q374, i.e., GST-D2R-IL3(M281-P310) and GST-D2R-IL3(P308-K340), did not precipitate the kinase. Thus, the C-terminal 30 amino acids (Q345-Q374) of D2R-IL3 contain a core binding region (Fig. 2C). Notably, a GST protein containing the corresponding region of IL3 in dopamine D3 receptors (D3R), GST-D3R-IL3(K346-Q375), failed to precipitate CaMKIIξ (Fig. 2B).

CaMKIIξ interacts with D2Rs in striatal neurons. CaMKIIξ and D2Rs are densely expressed in striatal neurons. To determine whether native CaMKIIξ and D2R interact with each other in these neurons, communoprecipitation was performed using synaptosomal samples extracted from the adult rat striatum. In proteins immunoprecipitated by anti-D2R antibodies, we readily detected immunoreactivity of CaMKIIξ (Fig. 3A). In a reverse communoprecipitation assay, we also observed a D2R band in the CaMKIIξ precipitates (Fig. 3B). Full-length blots were presented in Supplementary Figure1. Thus, endogenous CaMKIIξ and D2R proteins in striatal neurons form complexes in normal animals with extracorporeal detection.

Effects of chronic levodopa administration on CaMKIIξ-D2R interactions. In this study, we investigated the effect of chronic levodopa injection on CaMKIIξ-D2R interactions in striatal neurons of 6-OHDA lesioned PD rats. We found that chronic levodopa administration (25 mg/kg, i.p.; twice daily for 22 days) produced an increase in an amount of D2Rs in CaMKIIξ precipitates as compared to control PD rats (Fig. 4A and 4B). This indicates an increased rate of CaMKIIξ-D2R interactions in striatal neurons following chronic administration of levodopa.

Based on the CaMKIIξ binding region in D2R-IL3 identified above, we synthesized a Tat-fusion interfering peptide (Tat-D2Ri) that contains a Tat cell membrane transduction domain (YGRKKRRQRRR) and a core region of CaMKIIξ binding motif (TMSRKKLSQKKEK) on D2R-IL3 (Fig. 4A). The arginine-enriched Tat domain renders cell permeability and the CaMKIIξ binding motif may compete with endogenous D2Rs for CaMKIIξ binding. Indeed, Tat-D2Ri locally injected into the rat striatum (1 μg/0.5 μl, 1 h prior to levodopa) at the final day of chronic levodopa treatment (25 mg/kg, i.p.; twice daily for 22 days) reduced the CaMKIIξ-D2R interaction in the injected site (Fig. 4A and 4B). In contrast to Tat-D2Ri, Tat-D2Rc (a sequence-scrambled control) did not alter the increased CaMKIIξ-D2R complex formation. Full-length blots were presented in Supplementary Figure2. These results validated the efficacy and selectivity of Tat-D2Ri in disrupting CaMKIIξ-D2R interactions in striatal neurons of adult rat brains in vivo. A single intrastriatal injection of the peptide was able to reverse the elevation of CaMKIIξ-D2R interactions induced by chronic levodopa administration.

Effects of Tat-fusion peptides on behavioral responses to levodopa. A series of neurobehavioral experiments were carried out to test the effect of Tat-fusion interaction-dead peptides on...
behavioral activities induced by levodopa. Chronic administration of levodopa induced a gradual increase in ALO AIM scores in PD rats as tested at 2, 7, 14, and 21 days after levodopa administration (Fig. 5A), indicating a development of LID. Interestingly, at day 22, intrastral infusion of Tat-D2Ri (1 μg/0.5 μl) alleviated these dyskinetic behavioral changes (Fig. 5B). In contrast, intrastral infusion of Tat-D2Rc or saline had no effect on dyskinetic behaviors. Chronic levodopa injection also shortened the duration of dyskinesia (Fig. 5C and Fig. 5D). Tat-D2Ri but not Tat-D2Rc significantly reversed this reduction. Detailed data were displayed within tables in supplementary information (Table 1 and Table 2).

**Discussion**

In this study, we investigated the possible interaction between CaMKIIα and D2Rs. We found that CaMKIIα bound to D2Rs in vitro. This binding appeared to be direct since a specific region in the D2R-IL3 domain formed a binding site for CaMKIIα. Two native proteins were also found to interact with each other in neurons. Based on the coimmunoprecitation data, CaMKIIα was found to form complexes with D2Rs in adult rat striatal neurons. We then set forth to investigate whether the CaMKIIα-D2R interaction in striatal neurons responds to levodopa therapy in a rat 6-OHDA lesioned PD model. We found that chronic levodopa administration induced a significant increase in the CaMKIIα-D2R interaction. A cell permeable Tat-D2Ri peptide injected into the striatum blocked this increase. Tat-D2Ri peptides also reduced the dyskinetic behavior induced by levodopa. These results indicate that D2Rs are a new interacting partner of CaMKIIα in striatal neurons. This newly identified CaMKIIα-D2R interaction may play a role in LID.

An important finding in this work is the interaction between CaMKIIα and D2Rs. CaMKIIα was found to bind to an intracellular domain of D2Rs. Specifically, CaMKIIα bound to IL3 but not IL2 and CT regions. A small distal region in the C-terminal IL3 forms a site accepting CaMKIIα. Dopamine D3 receptors have also been found to be bound by CaMKIIα. However, interestingly, CaMKIIα binds to the N-terminal region of D3R IL3 as opposed to its binding to the C-terminal region of D2R IL3. At present, we have not carried out experiments to fully define the functional role of CaMKIIα-D2R interactions in regulating D2R expression and function. As a G protein-coupled receptor, D2Rs are couple to Gαi proteins, through which D2Rs inhibit adenylyl cyclase and reduce the downstream cAMP formation. IL3 is a known region that couples D2Rs to Gαi proteins. Within IL3, the C-terminal membrane-proximal part participates in the interaction with Gαi. Noticeably, the CaMKIIα binding motif we identified in D2R-IL3 partially overlaps with the Gαi interaction domain. Thus, CaMKIIα is assumed to have an impact on the D2R-Gαi-adenyl cyclase signaling pathway. This topic will be investigated in the future.

The basal ganglia circuitry contains a D1 receptor-bearing direct pathway and a D2R-bearing indirect pathway. An essential pathophysiological characteristic of LID is the presence of hypoactivity of the indirect pathway and hyperactivity of the direct pathway. Although D2Rs are considered to be important to LID, direct evidence is incomplete in illustrating the role of D2Rs. Thus, in this study, we attempted to investigate the role of D2Rs in terms of its interactions with CaMKIIα. The important findings obtained in this study include that 1) chronic levodopa administration increased the CaMKIIα-D2R interaction in the striatum of PD rats, 2) This increase was disrupted by an interaction-dead peptide (Tat-D2Ri) but not by a control peptide, and 3) more importantly, Tat-D2Ri showed the ability to reduce dyskinetic behavioral responses to levodopa. These results collectively indicate that the CaMKIIα-D2R interaction in striatal neurons is an important pathway to LID. This interaction was upregulated as a plastic response to long-term levodopa therapy, which may contribute to dyskinetic behavior seen in LID rats. Co-administration of partial D2R agonists with levodopa attenuated levodopa-induced abnormal behavioral response. Thus, if CaMKIIα exerts an inhibitory regulation of D2Rs, Tat-D2Ri, by removing the CaMKIIα influence, can disinhibit D2Rs and achieve the same effect as that induced by D2R agonists. Consistent with this model, intrastral injection of CaMKII inhibitors improved motor performance and synaptic plasticity in the form of long-term potentiation at corticostriatal synapses. Moreover, intrastral injection

![Figure 2](image-url) **The CaMKIIα binding motif in D2R-IL3.** (A) Binding of CaMKIIα to GST-fusion proteins derived from D2R-IL3. (B) Binding of CaMKIIα to GST-fusion proteins derived from D2R-IL3 and D3R-IL3. Note that Q345-Q374 fragments showed the ability to bind to CaMKIIα. (C) Amino acid sequence of D2R-IL(Q345-Q374). *In vitro* binding assays were carried out with purified recombinant proteins. Bound CaMKIIα was visualized by immunoblots (IB) of the eluted proteins using a specific antibody.

![Figure 3](image-url) **Interactions of CaMKIIα with D2Rs in adult rat striatal neurons.** (A) Coimmunoprecipitation of endogenous D2Rs and CaMKIIα using an anti-D2R antibody as the immunoprecipitating antibody. (B) Coimmunoprecipitation of endogenous D2Rs and CaMKIIα using an anti-CaMKIIα antibody as the immunoprecipitating antibody. Coimmunoprecipitated proteins were visualized by immunoblots (IB) of the eluted proteins using antibodies indicated. Full-length blots were presented in Supplementary Figure 1.
Expression and purification of glutathione S-transferase (GST)-fusion proteins. cDNA fragments encoding intracellular domains, such as intracellular loops (IL) and C-termini (CT), were generated by polymerase chain reaction amplification from full-length cDNA clones. These fragments include D2R-IL2 (D131-R151), D2R-CT (N41-C444), D2R-IL3 (K211-Q374), D2R-IL3 (K211-M270), D2R-IL3 (G242-Q374), D2R-IL3 (M281-P310), D2R-IL3 (P308-K340), D2R-IL3 (Q345-Q374), and D3R-IL3 (K346-Q375). These fragments were subcloned into BamHI-EcoRI sites of the pGEX-4T-3 plasmid (Amersham Biosciences, Arlington Heights, IL) or SpeI-XhoI sites of the pET-41a (+) plasmid (Novagen, Madison, WI). To confirm appropriate splice fusion, all constructs were sequenced. GST-fusion proteins were expressed in E. coli BL21 cells (Amersham) and purified from bacterial lysates as described by the manufacturer. His-tagged full-length CaMKIIα (M1-H478) was expressed and purified via a baculovirus/Sf9 insect cell expression system.

Affinity purification (pull-down) assay. Pull-down assays were performed with solubilized striatal extracts (50–100 μg of protein) diluted in 1X phosphate-buffered saline (PBS)/1% Triton X-100 and incubated with 50% (v/v) slurry of glutathione-sepharose 4B beads (Amersham). Assay solutions were saturated with GST alone or the indicated GST-fusion protein (5–10 μg) for 2–3 h at 4°C. After beads were washed four times with 1X PBS/1% Triton X-100, bound proteins were eluted with 2X lithium dodecyl sulfate (LDS) loading buffer, resolved by SDS-PAGE, and immunoblotted with a specific antibody.

In vitro binding assay. To conduct binding assays, GST-fusion proteins (1–5 μg) were digested with 0.2 NIH unit of thrombin (Amersham) for 2 h at room temperature. The reaction was stopped by phenylmethylsulfonyl fluoride (10 μM). GST was removed by glutathione sepharose (Amersham). The supernatant was equilibrated to binding buffer (200 mM NaCl, 0.2% Triton X-100, 0.1 mg/ml BSA, and 50 mM Tris, pH, 7.5) with 0.5 mM CaCl₂ and 1 μM CaM. Binding reactions started after adding GST-fusion proteins and remained for 2–3 h at 4°C. To precipitate GST-fusion proteins, 10% glutathione sepharose was added. The precipitate was washed three times with binding buffer. Bound proteins were eluted with 2X LDS loading buffer, resolved by SDS-PAGE, and immunoblotted with a specific antibody.

Animals and 6-OHDA lesions. Animal experimental design was seen in supplementary figure3. Adult female rats (Sprague Dawley, 180–220 g, Sippr-BK Ltd, Shanghai, China) were used in this study. All procedures were carried out in accordance with guidelines of the National Institutes of Health for the care and use of laboratory animals. Rats were deeply anesthetized by 7% chloral hydrate (0.5 ml/kg) and mounted in a stereotaxic apparatus (Narishge, Japan) equipped with a rat adaptor. Animals received unilateral injections of 6-OHDA (8 μg) or saline (sham lesion) into the right medial forebrain bundle (MFB) of the rat brain (mm): AP = −4.4 mm from bregma; ML = −1.2 mm from midline; DV = −7.8 mm from the dura surface; Tooth bar = −2.4 mm. A volume of 4 μl was injected over 4 min and the Hamilton syringe was kept in place for an additional 5 min before being retracted slowly. At 3 weeks after surgery, rats were tested with a subcutaneous injection of apomorphine at 0.05 mg/kg (WOKO, Japan). Contralateral turning was counted for 30 min after an interval of 10 min. Only those rats displaying rotational asymmetry of >6 turns/min were considered as PD rats and were used for the following neurochemical and behavioral experiments.

Drug treatment. Validated PD rats received vehicle or levodopa injection for 22 days. Vehicle or levodopa methylester (Sigma, St. Louis, MO) was given intraperitoneally (i.p.) at 25 mg/kg in combination with benserazide (6.25 mg/kg). Animals were treated with levodopa and benserazide twice daily (6 h apart). At the final day (day 22), levodopa-treated and dyskinetic rats were randomly divided into 3 groups. These rats received intrastriatal administration of Tat-D2Ri, Tat-D2Rc, or saline. One hour after fully recovery from anesthesia, rats were treated with levodopa and benserazide. For intrastratial injection, rats were anesthetized by 7% chloral hydrate (0.5 ml/100 g, v/w). A volume of 0.5 μl Tat-D2Ri (1 μg) or Tat-D2Rc (1 μg) or saline was injected at the coordinates: AP = 0.5 mm from bregma; ML = −2.5 mm from midline; and DV = −4.2 from the dura surface. A microsyringe was kept in place for an additional 5 min before being retracted slowly. Rats were left on a warm plate after surgery to avoid hypothermia until recovery.

Behavioral test. The evaluation of AIM was performed according to the rat dyskinesia scale[30]. On testing days, rats were placed individually in transparent plastic cages 10 min before drug treatment. As described previously[4, 5], rat abnormal involuntary movements (AIM) were classified into four subtypes: axial AIM, i.e. dystonic posturing or choreiform twisting of the neck and upper limbs towards the side contralateral to the lesion; limb AIM, i.e. abnormal, purposeless movements of the forearm and digits contralateral to the lesion; orolingual AIM, i.e. empty jaw movements and contralateral tongue protrusion; and locomotive AIM, i.e. increased locomotion with contralateral side bias. Each of these subtypes was scored on a severity scale from 0 to 4. During a period of 180 min following levodopa treatment, three subtypes of AIM were assessed as axial, limb, orolingual every 20 min (60 sec monitoring period for each). The ALO AIM were tested at 2, 7, 14 and 21 days during levodopa treatment. At day 22, rats were intrastratial injected with Tat-D2Ri, Tat-D2Rc or saline. One hour after fully revived from anesthesia, levodopa was administered and behavioral assessments were then carried out. The response duration was also recorded which was defined previously[4, 5]. The duration of the rotational response was measured as the time between the first 5 min interval when turning exceeded 20% of the peak rate and the first interval when turning fell below...
20% of the peak rate. The peak intensity of rotation was measured as the peak number of contralateral turns in any 5 min interval.

Coimmunoprecipitation and immunoblot. Rat striatal tissue was dissected on ice and homogenized by sonication in an immunoprecipitation lysis buffer (Beyortime, China) plus a protease inhibitor cocktail (Roche Diagnostics, Swiss). To obtain P2 pellets, the homogenate was centrifuged at 800 g for 10 min at 4 °C. Membranes were suspended in a buffer containing 0.5% SDS and boiled for 5 min. Samples were loaded on 5–10% SDS gels. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% nonfat milk for 1 h at room temperature and incubated with a rabbit primary antibody against D2Rs (Millipore) or CaMKII protein kinase. The complex was precipitated with protein G agarose beads or protein A agarose beads by gentle rocking for 3 h at 4 °C. The supernatant was washed and incubated in horseradish peroxidase conjugated secondary antibodies (Santa Cruz) overnight at 4 °C. Membranes were blocked in 5% nonfat milk for 1 h at room temperature and incubated with a mouse primary antibody against CaMKII (Santa Cruz) overnight at 4 °C. Membranes were washed and incubated in horseradish peroxidase conjugated secondary antibodies (1: 1000) for 1 h at room temperature. Immunoblots were developed with the enhanced electrochemiluminescence reagent (GE Healthcare) and captured by a BIO-RAD molecular imager.

Data analysis. The O.D. value of blots bands were measured by image lab™ software and normalized with PD control. SPSS17.0 and Graphpad prism5 were used for stastics and Graphics. Results of behavioral tests are presented as means ± SD. The data were evaluated using a one-way ANOVA followed by a Bonferroni’s comparison of groups using least-squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

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Figure 5 | Effects of Tat-D2Ri and Tat-D2Rc on dyskinetic behaviors induced by levodopa. (A) Effects of chronic levodopa administration on ALO AIM scores. (day2 vs day21, lid + saline group: p = 0.03; lid + Ri group: p = 0.00; lid + Rc group: p = 0.03) (B) Effects of Tat-fusion peptides on ALO AIM scores at day 22. (lid + saline vs lid + Ri: p = 0.049; lid + Ri vs lid + Rc: p = 0.025; lid + Rc vs lid + saline p = 1.000) (C) Effects of chronic levodopa administration on the duration of response. (day2 vs day21, saline group: p = 0.00; lid + Ri group: p = 0.00; lid + Rc group: p = 0.00) (D) Effects of Tat-fusion peptides on the duration of response at day 22. (lid + saline vs lid + Ri: p = 0.027; lid + Ri vs lid + Rc: p = 0.044; lid + Rc vs lid + saline p = 1.000). Note that chronic levodopa administration increased ALO AIM scores and reduced the duration of rotation. Tat-D2Ri can significantly reverse these behavioral changes. Levodopa was given to 6-OHDA lesioned PD rats at 25 mg/kg (i.p.; twice daily for 22 days). At day 22, Tat-D2Ri and Tat-D2Rc (1 μg) was infused bilaterally into the striatum 1 h before levodopa. Behavioral tests were conducted at 2, 7, 14, 21, and 22 days. p < 0.05 versus #day2 of the same group; * lid + saline group on the same day.
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**Author contributions**

L.Z.G. have contributed to the conception and design of the study. Z.S.F. performed the experiments, wrote the main manuscript text and prepared figures 3–5. W.Q. performed the experiments, prepared figures 1 and 2, and revised the article. X.C.L. participated in the statistical analysis. All authors have reviewed the manuscript.

**Additional information**

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