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·Original Article·

Extracellular signal-regulated protein kinase activation in spinal cord contributes to pain hypersensitivity in a mouse model of type 2 diabetes

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ABSTRACT

Painful peripheral neuropathy is a common complication of diabetes mellitus. The symptom of pain can become a major factor that decreases the quality of life of patients with diabetes, while effective treatment is lacking. In the present study, we aimed to investigate the changes of pain threshold in the early stage of diabetes in db/db mice, an animal model of type 2 diabetes mellitus, and the underlying molecular mechanisms. We found that (1) db/db mice (with a leptin receptor-null mutation and characterized by obesity and hyperglycemia) showed hypersensitivity to mechanical and thermal stimuli at the early stage of diabetes; (2) phosphorylated extracellular signal-regulated kinase (pERK), but not total ERK in the spinal cord and dorsal root ganglia in db/db mice significantly increased compared with wild-type mice. The increased pERK immunoreactivity occurred in both NeuN-expressing neurons and GFAP-expressing astrocytes, but not in Iba-1-expressing microglia; (3) both single and consecutive (for 5 days) intrathecal injections of U0126 (2 nmol per day), a selective MEK (an ERK kinase) inhibitor beginning at 8 weeks of age, attenuated the bilateral mechanical allodynia in the von-Frey test and heat hyperalgesia in Hargreave's test; and (4) db/db mice also displayed increased nocifensive behavior during the formalin test, and this was blocked by intrathecal injection of U0126. Also, the expression of pERK1 and pERK2 was upregulated following the formalin injection. Our results suggested that the activation of ERK in spinal neurons and astrocytes is correlated with pain hypersensitivity of the type 2 diabetes animal model. Inhibiting the ERK pathway may provide a new therapy for pain control in type 2 diabetes.

Keywords: painful diabetic peripheral neuropathy; db/db mice; pain hypersensitivity; extracellular signal-regulated kinases

INTRODUCTION

Diabetic peripheral neuropathy (DPN), one of the most common complications of diabetes mellitus, occurs in up to 20% of patients and in up to 50% of those with neuropathy[1]. The occurrence rises with age and duration of diabetes, and impairs the quality of metabolic control[2]. DPN, characterized by symmetric, distal paresthesia, affects 54% of patients with type 1 and 45% of those with type 2 diabetes. The symptom of pain, in 11–30% of patients with DPN, is termed painful diabetic peripheral neuropathy (PDPN) or diabetic peripheral neuropathic pain[3], which is defined by the International Association for the Study of Pain as "pain arising as a direct consequence of abnormalities in the peripheral somatosensory system

in people with diabetes"[4]. Patients with PDPN often experience allodynia (painful responses to normally innocuous stimuli) and hyperalgesia (an increased responsiveness to noxious stimuli). The PDPN symptoms are usually described by patients as prickling, like an electric shock, burning, or sharp, and these are often exacerbated at night[5]. The painful condition can persist for a long time and greatly decreases the quality of life[6, 7]. Current treatment strategies for PDPN include blood glucose control, anticonvulsants, antidepressants, opioid analgesics, and combinations of these. However, only a few patients receive effective pain relief. Moreover, these drugs are limited by their side-effects and the risk of drug interactions[8, 9]. Therefore, it is necessary to understand the pathogenic mechanisms of PDPN in order to develop new therapeutic strategies.

Animal models of type 1 diabetes, especially streptozotocin (STZ)-induced diabetic rats and mice, have been extensively used to study PDPN for the past decades[10-12]. Meanwhile, information about the pathogenic mechanisms of PDPN in models of type 2 diabetes is limited, despite the fact that patients with this form are in the majority. There are predicted to be >200 million patients with type 2 diabetes worldwide by 2025[13]. Further, PDPN is more common and continues for a longer period in type 2 than in type 1 diabetes[14,15]. It is therefore vital to explore the mechanisms of PDPN of type 2 diabetes and find more effective treatments. The C57BLKS db/db mouse is a well-characterized model of type 2 diabetes mellitus due to its defective leptin receptor[16]. Similar to patients with type 2 diabetes, db/db mice present with obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia, and are widely used in research on diabetic complications.

Extracellular signal-regulated kinases (ERKs), including ERK1 (44 kDa) and ERK2 (42 kDa), with p38 and c-Jun N-terminal kinase (JNK), constitute the family of mitogen-activated protein kinases. Accumulating evidence indicates that ERK is involved in physiological and pathological conditions through its role in synaptic plasticity in the central nervous system[17-23]. Previous studies indicated that ERK activation in the spinal dorsal horn plays an important role in the development and maintenance of mechanical allodynia under type 1 diabetic conditions. Intrathecal administration of U0126, an inhibitor of MEK (an ERK kinase), reverses the pain hypersensitivity in diabetic rats[10,11]. However, little is known about the possible involvement of ERK activation in type 2 diabetes mellitus.

In the present study, we investigated (1) whether ERK is activated during diabetic pain at the early stage in db/db mice; and (2) whether the activation contributes to the pain hypersensitivity developed in diabetic mice. In addition to the degenerative changes, diabetes is also associated with a chronic inflammatory response characterized by abnormal cytokine production, increased acute reactions, and activation of inflammatory signaling pathways. Therefore, in this study we also investigated the behavioral response to a chemical inflammatory challenge in PDPN in type 2 diabetes.

MATERIALS AND METHODS

Animals

Diabetic mice (db/db) and non-diabetic littermates (wild-type, WT) of the C57BLKS strain were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). They were housed at 22 ± 2°C, with 55 ± 15% humidity under a 12-h light/dark cycle, and fed normal mouse chow in air-filtered cages with food and water available *ad libitum*. All experiments were approved by the Shanghai Animal Care and Use Committee. This study complied with the Ethical Standards of the International Association for the Study of Pain[24].

Drug Administration

U0126 was purchased from Sigma (St. Louis, MO), dissolved in dimethylsulfoxide (DMSO, Sigma) at 1 mg/mL, and then diluted in phosphate buffered saline (PBS) to the appropriate concentrations. To minimize inflammatory responses to intrathecal surgery, the reagents were delivered by spinal puncture with a 30-gauge needle between the L5 and L6 vertebrae[27]. Briefly, the mice were anesthetized with 1.5% isoflurane in air *via* a gas delivery system (TEC 3; Medical Supplies & Services Int. Ltd, West Yorkshire, UK). A brisk tail-flick was observed immediately after the needle entered the subarachnoid space. U0126 or vehicle was injected intrathecally in a total volume of 5 μL with a 10-μL Hamilton syringe.

Behavioral tests were performed at 1, 3, 6 and 24 h after single injections of drug/vehicle. For multiple

injections, the mice received daily intrathecal injection of U0126 (2 nmol) for five consecutive days starting on the first day at 8 weeks of age. Behavioral tests were carried out on day 1, day 3, and day 5 after the first U0126 injection. In the formalin test, mice were pretreated intrathecally with the antagonist 30 min before formalin hindpaw injection. The dose of U0126 was selected based on previous studies[28].

Blood Glucose and Body Weight

Blood glucose and body weight were measured at 08:00 to 09:00 on the first day of each week. Blood samples were collected by tail-prick from 10 mice in each group. To confirm the onset of diabetes, blood glucose was measured using a standard glucometer (Accu-Check Integra test strips, Roche, Germany) from 4 weeks of age. The body weight was measured with an electronic weighing scale.

Behavioral Tests

***von* Frey test for mechanical allodynia** The mechanical threshold was measured by probing *von* Frey filaments (*von* Frey hairs, Stoelting, Wood Dale, IL) with a protocol similar to that described by Bao *et al.*[25]. Briefly, each mouse was placed in a chamber (10 × 10 × 20 cm^3) with a customized platform that contained 1.5-mm diameter holes in a 5-mm grid of perpendicular rows throughout its entire area. Mice were allowed to acclimate for ~30 min. A series of *von* Frey filament stimuli (0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 g) were delivered to the central region of the plantar surface of the hindpaw in an ascending sequence until hindpaw withdrawal. Each filament was applied five times, for 2 s each time, at 15-s intervals. The paw-withdrawal threshold (PWT) was defined as the gram value of the filament to which the mouse responded with hindpaw withdrawal in at least three of the five applications.

Hargreaves' test for thermal hyperalgesia As previously described[25], the mice were placed individually in transparent plastic chambers on an elevated glass surface. After acclimation to the test chamber for 30 min, a radiant heat source (model 336 combination unit, IITC/Life Science Instruments, Woodland Hill, CA) was focused on the hindpaw. The heat source was turned off when the mouse lifted the foot. The hindpaw-withdrawal latency (PWL) was defined as the time from the onset of radiant heat application to the withdrawal of the hindpaw. To prevent tissue damage, the cut-off latency was set at 20 s. The average of three trials was determined and the interval between trials was 10 min.

Formalin test Mice were habituated to plastic chambers on a room-temperature glass surface for 30 min. After intraplantar (right hindpaw) formalin injection (2.5%, 20 μL in 0.9% NaCl), the mouse was immediately returned to the chamber. The time of lifting and licking was recorded in each 5 min for a total of 1 h. The formalin score was calculated with a slight modification[26]: Formalin score = $(Time_{lifting} + Time_{licking})/300$.

Western Blot Analysis

Before and 1 h after formalin injection, both db/db and WT mice were deeply anesthetized with 25% urethane (1.5 g/kg body weight) at 8 weeks of age and the lumbar spinal cord and DRGs were quickly removed. The collected tissues were homogenized in lysis buffer (12.5 μL/mg tissue) containing PMSF (Sigma) and a mixture of protease inhibitors cocktail (Roche, Diagnostics Ltd, Mannheim, Germany). Protein samples were resolved by SDS-PAGE electrophoresis (10% gel, Bio-Rad), and transferred onto PVDF membranes (Millipore, Billerica, MA). The membranes were incubated in blocking solution (5–10% non-fat milk dissolved in Tris-buffered saline containing 0.05% Tween-20, TBST) at room temperature for 2 h, and incubated overnight at 4°C with a primary antibody (rabbit anti-total ERK, 1:100 000, Sigma; or mouse anti-pERK, 1:2 000, Sigma). After washes, the membranes were incubated with the corresponding HRP-conjugated secondary antibody (goat anti-rabbit IgG, 1:5 000 or goat anti-mouse IgG, 1:1 000) at 4°C for 2 h. GAPDH was used as a loading control. Signals were finally visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL) and the bands were visualized with the ChemiDoc XRS system (Bio-Rad). The integrated optical density of the bands was analyzed with the Bio-Rad Image Analysis System. Experiments were repeated three times for each sample under the same conditions.

Immunohistochemistry

Mice were anesthetized with 25% urethane (1.5 g/kg) at 8 weeks of age and perfused transcardially with normal saline (0.9% NaCl) followed by ice-cold 4% paraformaldehyde in PBS (pH 7.4, 0.1 mol/L). Lumbar spinal cord samples were

removed, postfixed in the same fixative for 2 h at 4°C, and immersed in 10–30% gradient sucrose in phosphate buffer (PB, pH 7.4, 0.1 mol/L) for cryoprotection. Transverse spinal sections (35 μm) were cut on a cryostat (Leica, Germany) and processed for immunofluorescence. In brief, sections were blocked with 10% donkey serum in 0.01 mol/L PBS with 0.3% Triton X-100 for 2 h at room temperature and then incubated overnight at 4°C with a mixture of mouse anti-pERK (1:1 000, Sigma) and rabbit anti-GFAP (1:1 000, Abcam), rabbit anti-Iba1 (1:500, Wako), or rabbit anti-NeuN (1:3 000, Merck Millipore) antibodies in PBS containing 1% normal donkey serum and 0.3% Triton X-100. After 3×10 min rinses in PBS, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:200, Jackson) and rhodamine-conjugated donkey anti-rabbit IgG (1:200, Jackson) for 2 h at 4°C. After three washes in PBS, the sections were coverslipped with a mixture of 50% glycerin in PBS, and then observed with a confocal laser-scanning microscope (Olympus BX61, Fluoview FV1000, Japan).

Statistical Analysis

All values are expressed as mean ± SEM. Differences among means were analyzed using two-way ANOVA with age and genotype as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were performed with the two-tailed Student's *t*-test. The results of Western blot were analyzed using Student's *t* test if two groups (WT and db/db mice) were used. Student's paired *t*-test was used to determine the significance of results of Western blot between ipsilateral and contralateral following formalin injection. In all analyses, P <0.05 was assumed to be statistically significant.

RESULTS

db/db Mice Develop Mechanical Allodynia and Thermal Hyperalgesia in the Early Stage of Diabetes

Blood glucose and body weight were measured in both db/db and WT mice from 4 to 12 weeks old to monitor the development of type 2 diabetes. The db/db mice had higher levels of blood glucose (two-way ANOVA, $F_{(1,18)}$ = 3358.838, P <0.001) and increased body weight (two-way ANOVA, $F_{(1,18)}$ = 7173.342, P <0.001) compared with the WT mice (Fig. 1). The body weight was significantly higher in db/db mice throughout the period tested (Fig. 1A). At 12 weeks of age, the weight of db/db mice (50.48 ± 0.50 g) was over twice that of WT mice (23.43 ± 0.24 g). The blood glucose of db/db mice reached 12.70 ± 0.89 mmol/L at 4 weeks, peaked at 9 weeks, and remained at a high level thereafter (Fig. 1B). These data showed that db/db mice developed obesity and hyperglycemia from 4 weeks, which is similar to the features of type 2 diabetes.

Since db/db mice had symptoms similar to patients with type 2 diabetes mellitus, they may also exhibit behaviors indicative of PDPN. The response threshold to *von* Frey hair stimuli was significantly lower in db/db mice beginning at 6 weeks, suggesting increased sensitivity to mechanical stimulation (mechanical allodynia). The mechanical allodynia lasted for 5 weeks, and diminished at 11 weeks of age (Fig. 1C, two-way ANOVA, $F_{(1,14)}$ = 41.276, P <0.001). Similarly, a decreased response latency to radiant heat stimulation was detected in db/db mice from 5 to 10 weeks of age as compared with age-matched WT mice (Fig. 1D, two-way ANOVA, $F_{(1,18)}$ = 62.919, P <0.001). These data showed that db/db mice developed mechanical allodynia and thermal hyperalgesia at the early stage of diabetes, indicating the presence of PDPN.

Increased ERK Activation in Spinal Astrocytes and Neurons during the Period of Pain Hypersensitivity in db/db Mice

Phosphorylation of ERK is an indicator of its activation[17,29]. Activation of the ERK cascade in dorsal horn neurons of the spinal cord following peripheral noxious stimulation contributes to both short-term and long-term pain hypersensitivity[17-21]. Compared with WT mice, the basal expression of pERK1 (44 kDa) and pERK2 (42 kDa) in the spinal cord was increased in db/db mice at 8 weeks (pERK1, WT 1.05 ± 0.09 *vs* db/db 1.73 ± 0.09, Student's *t*-test, P <0.001; pERK2, WT 1.60 ± 0.18 *vs* db/db 2.19 ± 0.10, Student's *t*-test, P = 0.008) (Fig. 2A, B). Meanwhile, both pERK1 and pERK2 were markedly increased in the DRG of db/db mice (pERK1, WT 0.90 ± 0.19 *vs* db/db 3.03 ± 0.38, Student's *t*-test, P = 0.002; pERK2, WT 1.28 ± 0.29 *vs* db/db 2.89 ± 0.41, Student's *t*-test, P = 0.019) (Fig. 2C, D). However, no significant changes were observed in total ERK1 or ERK2 expression in the spinal cord and DRG between the two strains of mice. Although both pERK1

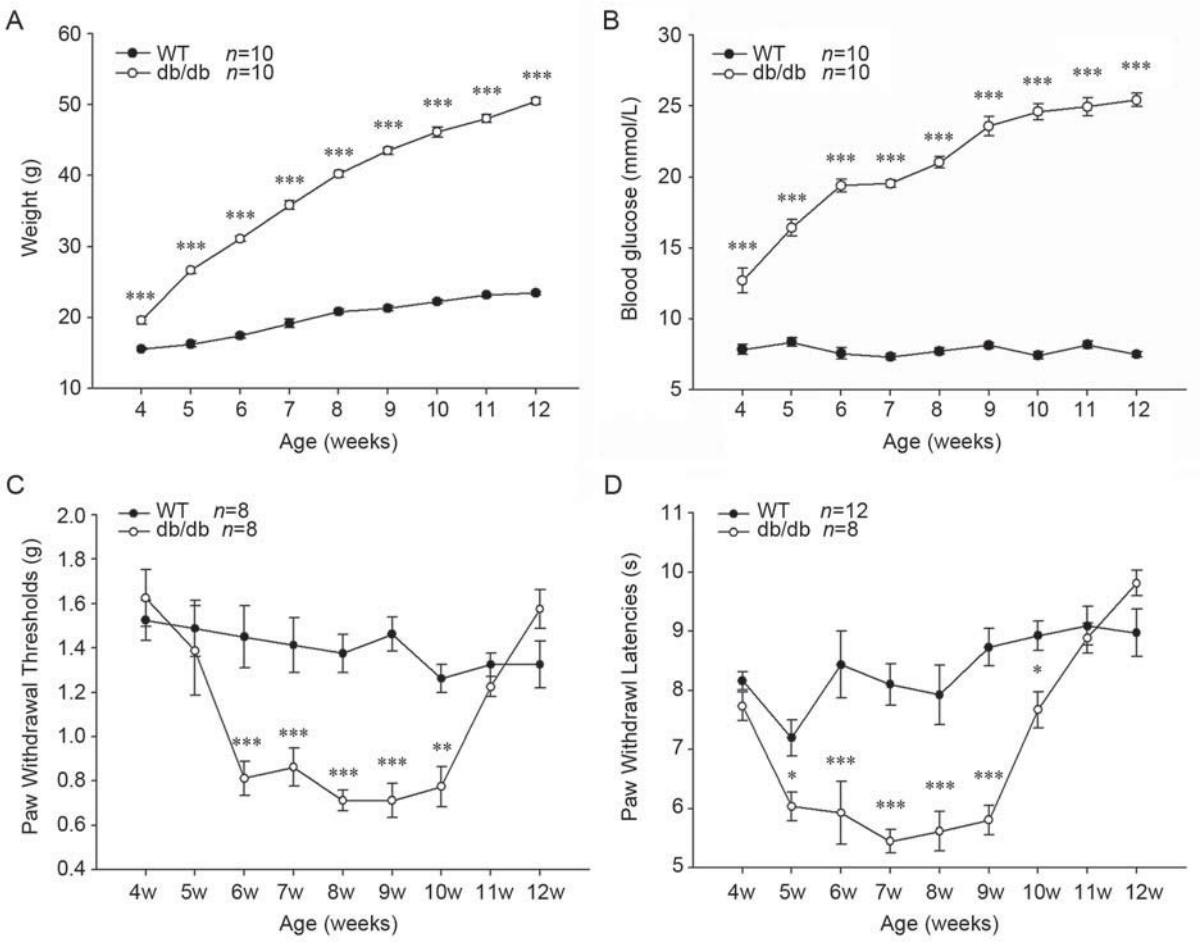

Fig. 1. db/db mice display obesity and hyperglycemia and develop mechanical allodynia and thermal hyperalgesia at an early stage. A and B: Features of type 2 diabetes in db/db mice. The body weight of db/db mice was significantly higher than WT mice beginning from 4 weeks of age, and lasting throughout the period tested (A). The blood glucose showed similar differences (B). C and D: Paw-withdrawal responses to mechanical and heat stimulation. The mechanical allodynia occurred at 6 to 10 weeks of age in db/db mice (C). The db/db mice displayed significantly reduced thermal thresholds compared to WT mice from 5 to 10 weeks of age (D). The behavioral data are averages of the left and right sides. *P <0.05, **P <0.01 and ***P <0.001 *vs* WT mice at the same age.

and pERK2 increased during the period of hyperpathia, the increase of pERK1 (1.6 fold in spinal cord and 3.4 fold in DRG) was much greater than that of pERK2 (1.4 fold in spinal cord and 2.3 fold in DRG), especially in the DRG, suggesting that pERK1 is the predominant form of pERK involved in PDPN in db/db mice.

To further identify the cell types that show spinal ERK activation, double immunofluorescence staining of pERK was performed with the cell-specific markers NeuN for neurons, GFAP for astrocytes, and Iba-1 for microglia. Immunoreactivity of pERK was predominantly co-localized with GFAP and less with NeuN (Fig. 2E–J). Besides, pERK did not co-exist with Iba-1. Our data indicated that the increased pERK in spinal astrocytes and neurons but not microglia plays a role in nociceptive behavior in db/db mice.

Intrathecal MEK Inhibitor Attenuates the Mechanical Allodynia and Thermal Hyperalgesia in db/db Mice

To determine the role of pERK in PDPN in db/db mice during the period of pain hypersensitivity, we suppressed the action of ERK with an MEK inhibitor, U0126, which does not affect the activity of other mitogen-activated protein kinases such as p38 and JNK. U0126 and vehicle were delivered into the L5–L6 CSF space by direct lumbar

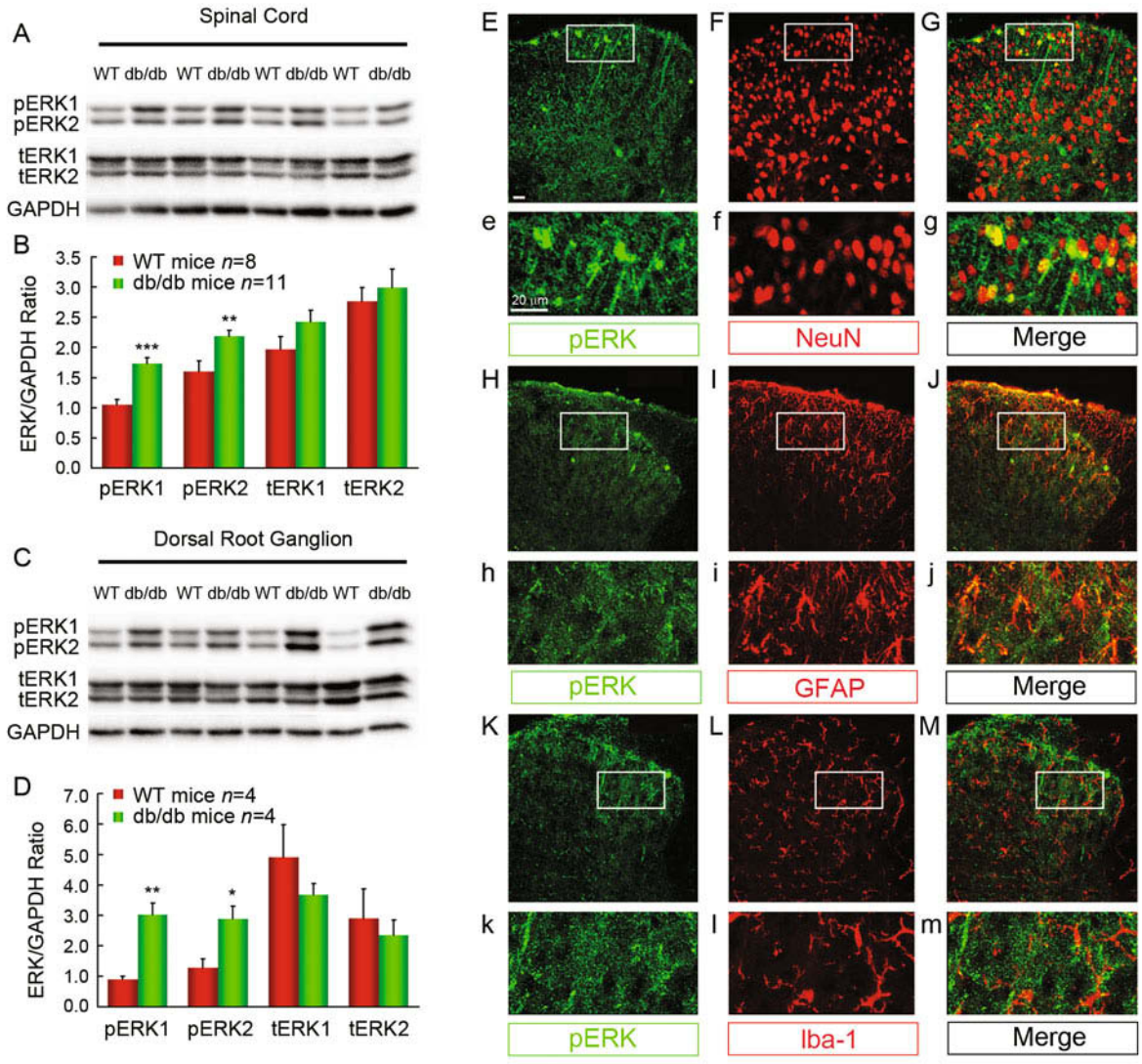

Fig. 2. Activation of ERK1/2 in spinal cord and DRG and the distribution of pERK in spinal cord at the early phase in db/db mice. A–D: Representative Western blots of pERK and total ERK in spinal cord and DRG and quantification. **P* <0.05, ***P* <0.01, ****P* <0.001 db/db mice *vs* WT mice. E–M: pERK (green) was predominantly co-localized with GFAP (astrocytic marker) (J, j), less with NeuN (neuronal marker) (G, g), and not with Iba-1 (microglial marker) (M, m). Scale bars, 20 μm.

puncture and paw-withdrawal responses to mechanical and thermal stimuli were tested before and 1, 3, 6 and 24 h after drug/vehicle administration. A single intrathecal injection of U0126 (2 nmol) significantly suppressed the mechanical allodynia (two-way ANOVA, $F_{(3,28)}$ = 54.114, P <0.001) and thermal hyperalgesia (two-way ANOVA, $F_{(3,28)}$ = 47.950, P <0.001) at 1 h and 3 h in db/db mice. Importantly, prolonged blockade of ERK activation by repeated intrathecal injection of U0126 (2 nmol) for 5 successive days starting from the first day at 8 weeks of age increased the paw-withdrawal threshold and latency in db/db mice. Even at 48 h after cessation of injection, the mechanical allodynia and thermal hyperalgesia were still reliably suppressed (Fig. 3B, D; two-way ANOVA: von Frey test, $F_{(1,14)}$ = 15.505, P <0.001; Hargreaves' test, $F_{(1,14)}$ = 20.017, P<0.001).

Enhanced Second Phase of Formalin Response in db/db Mice is Suppressed by Pretreatment with an MEK Inhibitor

To further explore the behavioral responses during

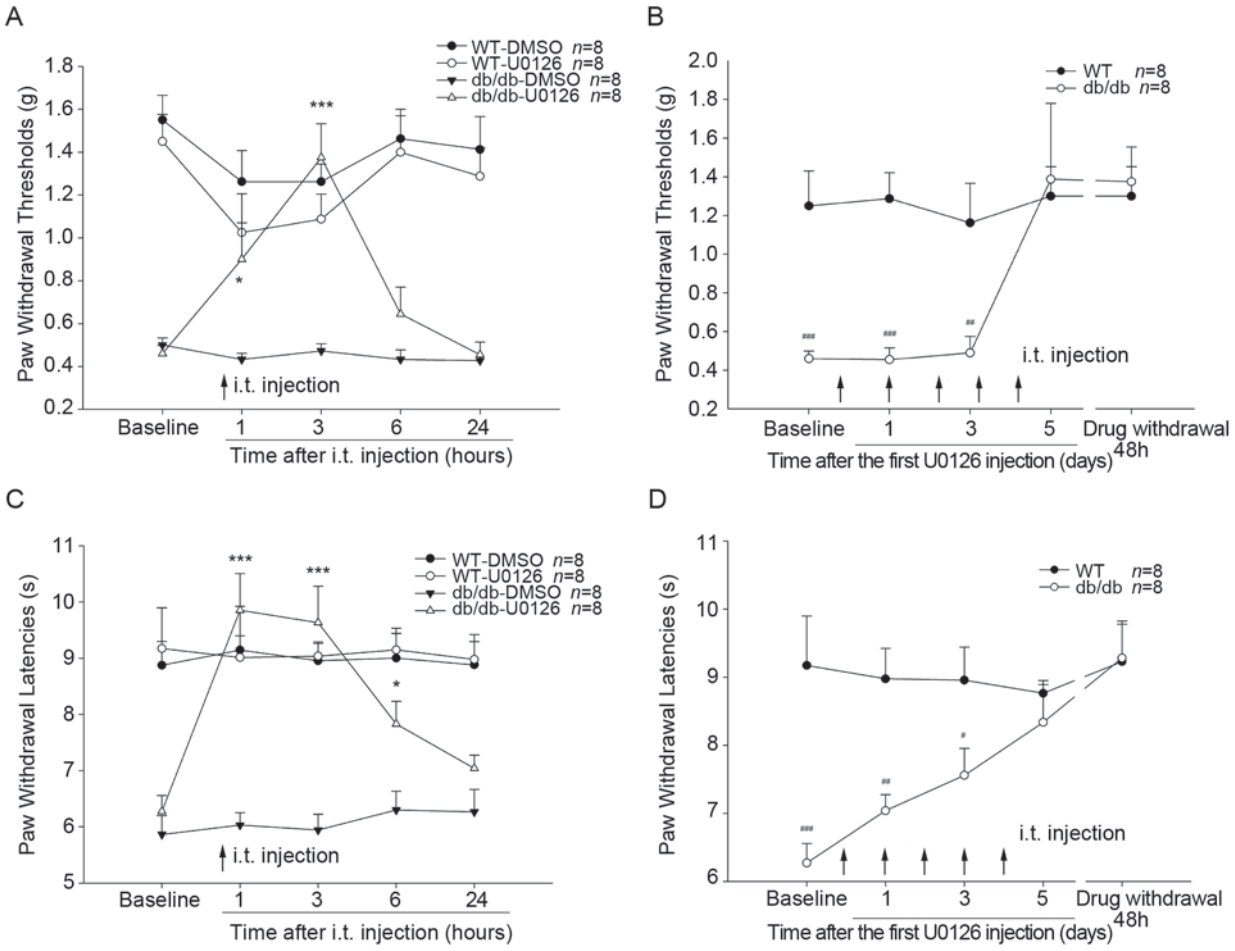

Fig. 3. Diabetes-induced mechanical allodynia and thermal hyperalgesia in db/db mice are reversed by intrathecal administration of the MEK inhibitor U0126 at 8 weeks of age. A and C, Mechanical allodynia and thermal hyperalgesia in db/db mice were suppressed at 1 h and 3 h after a single intrathecal injection of U0126 (2 nmol). ****P* <0.05, ******P* <0.001, db/db mice with U0126 *vs* db/db mice with DMSO. B and D, Repeated intrathecal injections of U0126 (2 nmol, once daily for 5 days) reversed the mechanical allodynia and thermal hyperalgesia in db/db mice. This effect lasted for at least 48 h after drug withdrawal. #*P* <0.05, ##*P* <0.01, ###*P* <0.001, db/db mice with U0126 *vs* WT mice with U0126.

inflammatory states in type 2 diabetes, we performed the formalin test, which is frequently used in studies of inflammatory pain in rodents[30]. Subcutaneous injection of 2.5% formalin (20 μL) into the hindpaw of mice resulted in a typical biphasic nociceptive response. Phase I begins immediately after injection and lasts for ~5 min; this is generally believed to involve the direct activation of nociceptors. After a short recovery defined as the quiescent phase (6–15 min), phase II begins at 15 min and lasts until 60 min after injection, although the duration and amplitude of this phase depend on the concentration of formalin used. Phase II of inflammatory nociception is thought to involve central as well as peripheral sensitization[31,32]. We therefore compared the responses of WT and db/db mice in the formalin test at 8 weeks of age. Compared with the WT mice, the quiescent phase (6–15 min) in db/db mice was increased (two-way ANOVA, $F_{(1,14)} = 7.034$, $P = 0.012$, Fig. 4). No significant difference in the early phase was observed (0–5 min, one-way ANOVA, $F_{(1,14)} = 1.588$, $P > 0.05$). But in phase II, the db/db mice showed a constant mildly-enhanced response of paw elevation compared to the WT mice, and a higher response peak. While an ANOVA comparing the traditionally-defined formalin phase II (16–60 min after injection) did not reach significance

(two way ANOVA, $F_{(1, 14)}$ = 0.445, P >0.05), a significant difference was found between WT and db/db mice when considering nociceptive behaviors 10–30 min after formalin injection (two-way ANOVA, $F_{(1, 14)}$ = 7.358, P = 0.008, Fig. 4). There was no significant difference between the two genotypes for the 31–60 min block.

Further, a single intrathecal administration of U0126 (2 nmol) 30 min before formalin injection significantly decreased phase II in db/db mice (two-way ANOVA, $F_{(1, 11)}$ = 10.046, P = 0.002, Fig. 4). These results suggested that the enhanced formalin-induced response in db/db mice requires ERK activation in the spinal cord, and further implicates ERK-mediated pain hypersensitivity in DPN of type 2 diabetes.

Increased pERK Expression in the Spinal Cord after Formalin Injection

We next investigated whether the enhanced sensitivity to formalin in the db/db mice was associated with increased ERK activation. ERKs play a central role in pain hyperalgesia in the spinal cord, so we assessed pERK in the two strains of mice after formalin injection. We found that after intraplantar (right hindpaw) injection, pERK1 and pERK2 in the ipsilateral cord were increased compared with those on the contralateral side in both db/db and WT mice (pERK1 in WT mice: contralateral 1.0 ± 0.19 *vs* ipsilateral 1.35 ± 0.18, P = 0.008; in db/db mice: contralateral 1.32 ± 0.23 *vs* ipsilateral 2.02 ± 0.30, P <0.001; pERK2 in WT mice: contralateral 1.0 ± 0.30 *vs* ipsilateral 1.37 ± 0.40, P = 0.048; in db/db mice: contralateral 1.18 ± 0.12 *vs* ipsilateral 1.60 ± 0.19, P = 0.004; all paired t-test) (Fig. 5A, B). Besides, the total ERK did not significantly differ between the two groups except for a slight upregulation in total ERK2 in the ipsilateral spinal cord of db/db mice compared to the contralateral side (Fig. 5C, D). Furthermore, ipsilateral ERK1 activation was higher in the db/db mice than in the WT mice (WT 1.4 ± 0.11 *vs* db/db mice 1.62 ± 0.11, Student's t-test, P = 0.041) (Fig. 5E, F), which suggests that pERK1 contributes more to the response induced by formalin in db/db mice.

DISCUSSION

ERK is an important player in inflammatory and neuropathic pain[33-36]. Here, we showed that ERK activation also

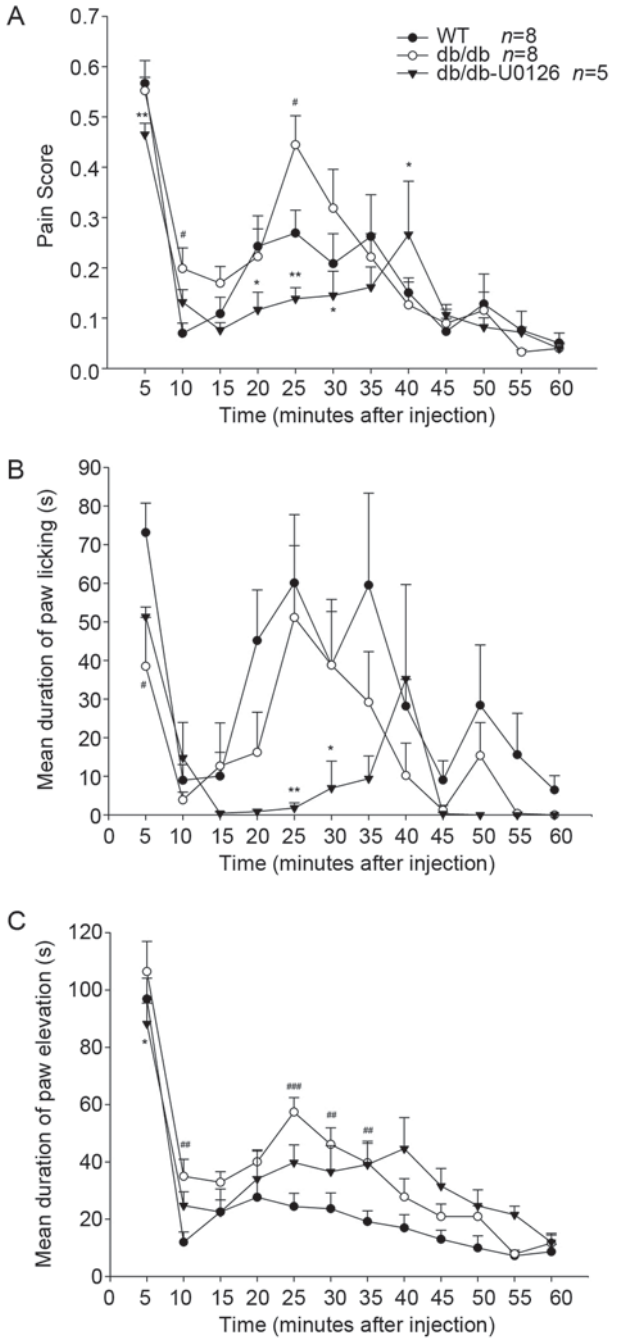

Fig. 4. Intraplantar injection of 2.5% formalin induces spontaneous pain. Pain score (A), paw licking (B), and paw elevation (C) in WT mice and db/db mice. Blockade of ERK activation with the MEK inhibitor, U0126, suppressed the enhanced responses during the formalin test in db/db mice. $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001, db/db mice *vs* WT mice (n = 8). $^{*}P$ < 0.05, $^{**}P$ <0.01 db/db mice without pretreatment (n = 6) *vs* db/db mice with U0126 (n = 5).

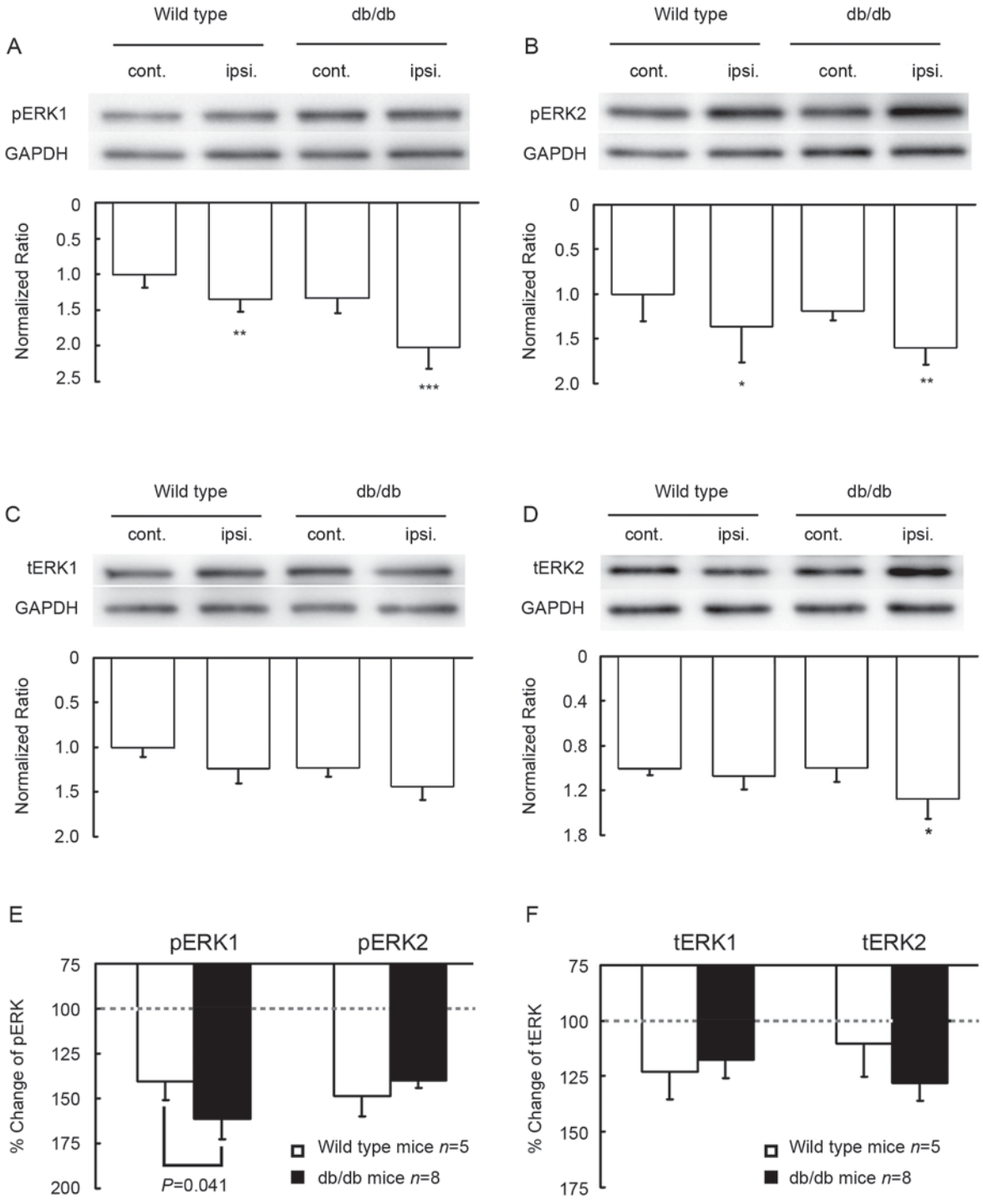

Fig. 5. Expression of ERK in the spinal cord of db/db and wild-type mice after formalin injection. A–D: Representative Western blots of pERK1/2 and total ERK1/2 in bilateral and contralateral spinal cord at 1 h after formalin injection. The histograms show the relative levels of pERK1, pERK2, total ERK1, and total ERK2, normalized to the contralateral side in WT mice. E and F: Changes of pERK1/2 and tERK1/2 on the ipsilateral side normalized to the contralateral side (100%). *P <0.05, **P <0.01, ***P <0.001 contralateral *vs* ipsilateral.

participates in pain hypersensitivity in db/db mice, a type 2 diabetes model. In this study, db/db mice developed mechanical allodynia and thermal hyperalgesia, features of PDPN at the early stage of diabetes. ERK phosphorylation was increased in the spinal cord and DRG at 8 weeks postnatal in db/db mice, and this activation occurred in both neurons and astrocytes in the spinal cord. And the MEK inhibitor U0126 reduced the pain hypersensitivity in the diabetic pain model. Further, the db/db mice displayed increased sensitivity to noxious formalin stimulation at 8 weeks of age along with elevation of pERK expression in the spinal cord. We therefore hypothesize that the different behavioral responses to mechanical, thermal, and noxious stimuli between db/db and WT mice may be related to abnormal sensory function, central sensitization, and elevated spinal pERK expression, especially pERK1.

In this study, the db/db mice developed obesity and hyperglycemia beginning at 4 weeks of age as well as transient mechanical allodynia and thermal hyperalgesia at 6–11 weeks of age. These are consistent with the findings by Cheng *et al.*[37,38] that db/db mice develop transient mechanical allodynia (beginning at 6 weeks and peaking at 8 weeks of age) in the early stage of diabetes. In contrast, Wright *et al.* reported that db/db mice display tactile hypoesthesia but normal thermal sensitivity after 6 weeks of age, and a decreased response to formalin in phase II at 16 weeks[39]. They suggested that the thermal thresholds are not altered because there is no quantitative deficit in epidermal or dermal innervation in the db/db mice, and argued that abnormal diabetes-induced thermal thresholds best reflect C-fiber damage. However, mounting evidence has suggested that, in addition to peripheral nerve damage, thermal sensitivity might be influenced by functional changes in DRG neurons and be modulated by central sensitization as well in different pathological states[40, 41]. Studies by Cheng and colleagues indicated that there are increased numbers of small-to-medium-sized nerve growth factor- and substance-P-immunopositive DRG neurons in db/db mice. We hypothesize that these increased pain transmitters or modulators might contribute to the pain hypersensitivity including thermal hyperalgesia in db/db mice. Another possible reason for these disparities may be the gender difference. It has been reported that female steroid hormones influence nociceptive behaviors, due to biochemical and functional alterations[42,43]. In the study of Wright *et al.*[39], the ratio of females to males was not presented and the author did not make a distinction between them. In the present study, all data were collected from male mice, and showed uniform nociceptive facilitation in different pain behavioral tests. Supporting evidence is that other animal models of type 2 diabetes, such as BBZDR/Wor rats, Zucker fatty rats, and ob/ob mice are reported to have similar pain behaviors[44-46]. Although patients with type 2 diabetes display diverse symptoms, including hyperalgesia, hypoalgesia, or insensitivity, our findings suggested that db/db mice model the subgroup of patients who develop pain hypersensitivity prior to the development of evident sensory neuropathy.

ERKs belong to the mitogen-activated protein kinase family and include ERK1 and ERK2. They were first found as primary effectors for growth-factor signaling. Then evidence demonstrated that activation of the ERK cascade is involved in neuronal plasticity, including pain hypersensitivity. ERK is activated after peripheral nerve injury, inflammation, and cancer in the spinal cord as well as the DRG[10, 11, 47]. It is widely accepted that pERK is only induced by high-threshold stimulation (activation of C- and Aδ-fibers) under normal conditions. However, after nerve injury or in some pathological states, low-threshold stimulation also causes ERK activation in neurons of the spinal dorsal horn[48,49]. To the best of our knowledge, we are the first to report that the basal pERK1/2 increased in PDPN without noxious stimulation. Interestingly, pERK1/2 activation was found not only in the dorsal horn but also in the DRG. Notably, the upregulation of activated ERK coincided with the development of mechanical allodynia and thermal hyperalgesia, suggesting that ERK plays an important role in mediating the pain in type 2 diabetes. Similarly, Daulhac *et al.* reported that ERK is significantly increased in the spinal cord and DRG at 3 weeks after STZ-induced diabetes in rats[10]. However, the underlying mechanisms remain to be elucidated, and additional work is required to verify the ability of insulin to reverse this cellular transduction pathway.

Previous studies have shown that the activation of ERK contributes to neuropathic and inflammatory pain in different cell types. For example, ERK phosphorylation occurs in spinal neurons and microglia after inflammation

and nerve injury, and then switches to astrocytes at late stages of neuropathic pain[50,51]. In the current study, we found pERK in astrocytes and neurons but not in microglia. Thus, the mechanism by which ERK is involved in diabetic pain seems quite different from that of neuropathic and inflammatory pain. However, Tsuda found that ERK is activated exclusively in microglia in the dorsal horn of STZ-induced diabetic rats, indicating that microglia play a crucial role in diabetes-induced disorders, mediated partly by the ERK signaling pathway[11]. Although it is difficult to explain the discrepancy between the two studies, the species difference might be primary. Previous studies have shown that the consequences in STZ-induced diabetic rats appear to differ from those in mice[52-56]. A recent study by Liao *et al.* indicated that spinal astrocytes are a vital component during DPN in type 2 diabetes[57]. They found that spinal astrocytes but not microglia are dramatically activated in db/db mice from postnatal weeks 4 to 20. Importantly, only the astrocyte-specific inhibitor *L*-α-aminoadipate but not the microglia-specific inhibitor minocycline attenuates mechanical allodynia in db/db mice[57]. Consistently, we found that activated ERK was predominantly expressed in astrocytes, suggesting their role in db/db mice. Alternatively, based on the behavioral results, we did not determine which types of cells had increased pERK1/2 immunoreactivity at the beginning (6 weeks) and the recovery (12 weeks) stages of pain hypersensitivity in db/db mice. Several studies have shown cellular shifts of ERK expression at different stages of pathological pain[34,58]. Therefore, we hypothesize that the cells with increased pERK1/2 immunoreactivity may be microglia or both neurons and glial cells at different time points.

Here, the MEK inhibitor U0126 was used to identify ERK activation. Both single and consecutive intrathecal administration of U0126 attenuated the mechanical allodynia and thermal hyperalgesia in db/db mice. This is consistent with reports from different groups showing that a single administration of U0126 reduces tactile allodynia and chronic treatment for 7 successive days suppresses mechanical hyperalgesia in STZ-induced diabetic rats[10,11]. Taken together, these results suggest that ERK activation contributes to the development and maintenance of abnormal pain during DPN in diabetes mellitus. Previous studies indicated that the suppressive effect of the MEK inhibitor may be through various downstream mechanisms[28,58-60]. Substantial evidence supports the hypothesis that the phosphorylation of N-methyl-*D*-aspartate receptors contributes to the central sensitization in the spinal cord after nerve injury or inflammation. U0126 inhibits the phosphorylation of the NR1 subunit (pNR1) and decreases mechanical hyperalgesia in diabetes-induced painful neuropathy; this suggests that pNR1 is regulated by ERK1/2[59]. In the present study, intrathecal U0126 attenuated the nociceptive behaviors in db/db mice, which implies that ERK1/2 might be essential for intracellular signaling that leads to the production of pro-nociceptive factors. However, the possible involvement of JNK and p38 in type 2 diabetes mellitus cannot be ignored. Also, the activator of ERK in db/db mice remains to be further investigated.

Studies in the past decade have demonstrated that the inflammatory response is triggered and maintained in type 2 diabetes and ultimately results in abnormal metabolic homeostasis[61,62]. In the present study, we also explored the response to inflammatory challenge (formalin injection) during the period of mechanical allodynia and thermal hyperalgesia in db/db mice. The formalin test is a commonly-used model of inflammatory (chemogenic) pain that elicits both an acute and a secondary pain stimulus. Our results showed that the nociceptive response of diabetic mice to formalin injection was significantly enhanced during the quiescent phase but not in phases I and II. Previous results with this test are diverse, probably due to the differences in formalin concentration used and the strain and species. Studies in STZ-induced diabetic rats indicated that a low concentration (0.5%) increases nociceptive behaviors in both the quiescent and the secondary phases, while a high concentration (5% formalin) only enhances the response in the quiescent phase[63-65]. In our study, 2.5% formalin was used and a significant increase during the quiescent phase (6–15 min) and the first half (16–30 min) but not the whole of phase II was observed. We therefore hypothesize that the concentration used in our study induced a moderate-to-severe nociceptive response in WT mice, such as to prevent increased pain behavior throughout phase II due to a “ceiling effect”. Another possibility is a difference between db/db transgenic mice and STZ-induced rats. Surprisingly, our results in db/db mice share similarities with other studies from transgenic mice in the formalin test. Two studies from Gereau’s lab showed that significant changes

only occur during the ascending part (first half) of phase II in both *Kv4.2*$^{-/-}$ mice and dominant-negative MEK mutant mice[28,66].

In summary, db/db mice, an animal model of type 2 diabetes, developed enhanced sensitivity to mechanical and thermal stimuli at the early stage of PDPN. The hypersensitivity was attenuated by the MEK inhibitor U0126. Increased ERK phosphorylation in spinal astrocytes and neurons played a role in diabetic pain. Moreover, db/db mice displayed increased nocifensive behavior during the formalin test and this was, in part, mediated by ERK. Therefore, inhibition of ERK may provide a new therapeutic candidate for PDPN in type 2 diabetes mellitus.

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