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ORIGINAL ARTICLE

Alterations of gene expression of sodium channels in dorsal root ganglion neurons of estrogen receptor knockout (ERKO) mice induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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Abstract Estrogen receptors (ERα and ERβ) mediate the neuroprotection of estrogens against MPTP-induced striatal dopamine (DA) depletion. Pain is an important and distressing symptom in Parkinson's disease (PD). Voltage-gated sodium channels in sensory neurons are involved in the development of neuropathic pain. In this study, MPTP caused changes in nociception and alterations of gene expression of voltage-gated sodium channels in dorsal root ganglion (DRG) neurons in ER knockout (ERKO) mice were investigated. We found that administration of MPTP (11 mg/kg) to WT mice led to an extensive depletion of DA and its two metabolites, αERKO mice were observed to be more susceptible to MPTP toxicity than βERKO or WT mice. In addition, we found that the mRNA levels of TTX-S and TTX-R sodium channel subtypes were differentially affected in MPTP-treated WT animals. The MPTP-induced up-regulation of Nav1.1 and Nav1.9, down-regulation of Nav1.6 in DRG neurons may be through ERβ, up-regulation of Nav1.7 and down-regulation of Nav1.8 are dependent on both ERα and ERβ. Therefore, the MPTP-induced alterations of gene expression of sodium channels in DRG neurons could be an important mechanism to affect excitability and nociceptive thresholds, and the ERs appear to play a role in nociception in PD.

Keywords Parkinson's disease · Pain · Estrogen receptor · Knock out · Voltage-gated sodium channels

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. The gradual, irreversible loss of dopamine (DA) neurons in the substantia nigra is the signature lesion of PD [1]. In addition to the motor disturbances experienced by the patients suffering from PD, several non-motor symptoms including pain also affect the PD patients. The recent surveys and case–control studies have been revealed that about 40–80% of patients with PD experience substantial pain [2–4]. PD experiencing pain can be described in different categories: musculoskeletal pain, radicular-neuropathic pain, dystonia-related pain, central neuropathic pain, and akathisia [4, 5]. However, the underlying mechanisms of pain in PD are unclear.

A greater prevalence and incidence of PD is reported in men than that in women. The ratio of men and women diagnosed with PD was around 2:1 [6], suggesting an involvement of sex hormones in the incidence of PD. 17β-Estradiol has been shown to protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that causes DA cell death in a similar way as the degeneration of DA neurons observed in PD [7, 8]. It is now well documented that estrogens produce its effects via estrogen receptors (ERs): ERα and ERβ. Moreover, using specific

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ERs agonists [9] and ERs knockout mice (ERKO) [10], it has shown that both ERs are necessary for protection.

It is clear that primary sensory neurons in the dorsal root ganglion (DRG) express ERs, but the role of these receptors in the mediating of the nociceptive system is not completely understood. Studies have shown that ERα is expressed only in the small, nociceptive neurons in the rat DRG while ERβ mRNA is expressed in all DRG neurons [11]. Estrogens acting primarily through ERβ may also have a developmental role that affects spinal cord structures, which are important for transmission of nociceptive information [12]. Furthermore, the selective activation of ERβ by ERb-131, a non-steroidal ERβ ligand, appears sufficient to provide beneficial effects in animal models of symptoms associated with neuropathic pain [13]. It is important to note that even in males, ERs may be involved in pain modulation as estrogen may produced by aromatization of testosterone [14].

The role of voltage-gated sodium channels in the transmission of nociceptive and neuropathic pain messages is well established. It is also increasingly clear that a number of drugs have usefulness in the treatment of neuropathic pain through a mechanism that involves block of voltage-gated sodium channels. DRG neurons can express two classes of Na^+ currents that can easily be separated pharmacologically on the basis of sensitivity to tetrodotoxin (TTX), one blocked by nanomolar TTX (TTX-S) and the other resistant to micromolar TTX (TTX-R). Adult DRG neurons can express the TTX-S sodium channels Nav1.1, Nav1.6, and Nav1.7, and the TTX-R sodium channels Nav1.8 and Nav1.9 [15].

This study was designed to investigate alterations in nociception after MPTP administration, and the role of the two ERs on nociceptive responses using ERKO. We also investigated that MPTP induced the gene expression properties of TTX-S and TTX-R sodium channels in acutely dissociated DRG neurons isolated from αERKO and βERKO mice.

Materials and methods

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the study was approved by the local IACUC. All efforts were made to minimize animal suffering and to reduce the number of animals used for the experiments.

Animals and treatments

C57Bl6 mice that were heterozygous for genomic deletion of ER types α and β (ERα and ERβ) were purchased from the Jackson Laboratory (B6.129P2-*Esr1*^{tm1Ksk}/J; B6.129P2-*Esr2*^{tm1Unc}/J). Inbreeding and appropriate backbreeding of the heterozygotes yielded homozygote knockout ERα (αERKO) and ERβ (βERKO) mice. The ERs genes were analyzed following the genotyping protocol of the Jackson Laboratory (BarHarbor, ME). Wild-type (WT) littermates were used as controls.

Mice were kept under environmentally controlled conditions (ambient temperature, 22°C; humidity, 40%) on a 12 h light/dark cycle with food and water ad libitum. Mice were equally distributed for age and weight in experimental groups of eight animals. Adult male WT [16], αERKO and βERKO mice (8–12 weeks, 21–28 g) were injected intraperitoneally four times with MPTP-HCl (11 mg/kg of free base; Sigma Chemicals, St. Louis, MO, USA) in saline at 2-h interval for 5 days [10, 17], while the control group received saline vehicle solution. All of the injections were given in a volume of 0.1 ml. On day 12, mice were sacrificed by decapitation. DRGs were isolated from acutely dissociated thoracic and lumbar regions of the spinal column, and frozen.

Measurement of striatal DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) levels

The left striatum were dissected, homogenized in ice-cold 0.1 M $HClO_4$ (100 µl/10 mg wet wt) containing 0.1 mM EDTA, and then centrifuged at 12,000 g for 20 min (4°C). The supernatant was filtered and stored at −70°C before assay.

The concentrations of DA and its metabolites DOPAC and HVA were measured by high performance liquid chromatography with electrochemical detection (HPLC-ECD). The Waters chromatographic system consisted of a 1525 pump, an automatic injector 717^+, and a Beckman column Cl8 reversed-phase, ODS-5 µm, 4.6 × 250 mm. The mobile phase was a 0.076 M Na_2HPO_4 buffer (pH = 3.9) containing 0.99 mM EDTA, 1.39 mM octyl-sodium sulfate, and 10% methanol (vol/vol). A mobile phase flow rate of 1.3 ml/min was used. Detections were made using an amperometric detector Waters 2465. The applied potential of the glassy carbon electrode was set at +600 mV relative to the Ag/AgCl electrode [16].

Behavioral testing

The latencies for thermal nociception were assessed using a radiant-heat tail-flick analgesia meter (IITC Scientific Products, Woodland Hills, CA) and the hot plate analgesia meter (IITC Scientific Products, Woodland Hills, CA). Radiant heat was focused on a spot 1–2 cm from the tip of the tail, and the latency until the mouse flicked its tail was recorded. The beam intensity was adjusted to give a tail flick latency of 3–4 s with a cutoff time of 15 s to avoid

tissue damage to the tail. The temperature in the hot plate test was 50 ± 0.2°C with a cut-off time of 60 s. The mice were placed on the hot plate confined by a lidded perspex box, and the latency to the first hind paw lick was recorded. The hot plate test was performed 1 min after the tail flick test. In the baseline nociceptive, two hot plate and three tail flick measurements were performed with a 15-min interval during the same day.

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from DRG neurons using the Trizol Reagent (Invitrogen Life Technologies, USA). Dry RNA pellets were dissolved in nuclease-free water, and DNAse-treated to minimize the risk of sample contamination. cDNA was reverse transcribed using 500 ng purified total RNA and PrimeScript™ RT reagent Kit (TaKaRa). Reverse transcription was performed in a 20-µl reaction mixture, at 37°C for 15 min and 85°C for 5 s. Quantitative real-time PCR was performed using 2 µl of sample cDNA on an ABI (Applied Biosystems) 7300 fast real-time PCR system using EvaGreen™ (Biotium, CA, USA) as a detection reagent. The cycling parameters were as follows: first 50°C for 2 min followed by DNA polymerase activation step at 95°C for 10 min and a two-temperature PCR of 40 cycles at 95°C for 15 s (denaturing step) followed by 60°C for 1 min (annealing step), and completed with a dissociation step for melting point analysis with 95°C for 15 s, 60°C to 95°C for 30 s, and 95°C for 15 s [18]. Data were calculated by $2^{-\Delta\Delta CT}$ and normalized to 18s rRNA, compared with those of control [19]. The sequences of the primers are listed in Table 1.

Statistical analysis

Data were expressed as means ± SEM for all the experiments. Student's *t* test were used as appropriate to evaluate the statistical significance of differences between two group means, and a one-way ANOVA with a Student–Newman–Keuls post hoc test was used for multiple comparisons. All tests of statistical significance were two-sided and the statistical significance was set at $P < 0.05$.

Results

Striatal biogenic amines

In the absence of MPTP, there were no significant differences between WT and αERKO or βERKO mice in striatal DA and its metabolites concentrations (Fig. 1). Administration of MPTP (11 mg/kg) to WT mice led to an extensive depletion of DA concentration (decrease of 45%) as well as DOPAC (decrease of 45%) and HVA (decrease of 68%). The degree of DA depletion was greater in the αERKO mice (17% relative to no MPTP) compared to WT mice (Fig. 1a). In contrast, there was no difference between WT and βERKO mice in the degree of DA depletion by the

Table 1 Sequence of primers

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)
Nav1.1	CAAAAAAGCCACAAAAGCCT	TTAGCTCCGCAAGAAACATC
Nav1.6	AGAAGAAGTACTACAACGCC	AGTAGTGTCTCAAGGCAAAC
Nav1.7	CAGCAAAGAGAGACGGAACC	CCCTCAGTGTCCGTAGAGATT
Nav1.8	AATCAGAGCGAGGAGAAGACG	CTAGTGAGCTAAGGATCGCAGA
Nav1.9	AGCCCAACGAAGTGAAGAAA	TCTCCAAGCCAGAAACCAAG
18s rRNA	CTTAGTTGGTGGAGCGATTTG	GCTGAACGCCACTTGTCC

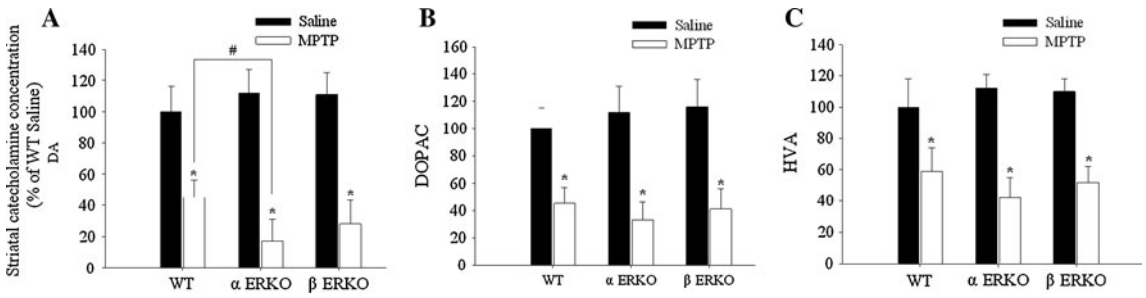

Fig. 1 Effects of treatment of MPTP on striatal DA and its metabolites (DOPAC and HVA) concentrations in WT, αERKO, and βERKO mice. Values are expressed as the percent of WT intact (saline vehicle solution) mice ± SEM of eight mice per group. *$P < 0.05$ versus respective intact, saline vehicle solution. #$P < 0.05$ versus WT mice treatment of MPTP

MPTP. Consistent with previous results [10], the αERKO mice showed a greater sensitivity to MPTP-induced DA depletion compared to the WT and βERKO mice.

Nociceptive tests

In agreement with previously published results [20], we observed that animals given MPTP showed a reduced tail flick latency compared to respective intact controls ($n = 8$, $P < 0.05$; Fig. 2a), there were no significant differences between WT and αERKO or βERKO mice ($P > 0.05$). The injection of MPTP also induced a reduced response latency in the hot plate test in the WT mice ($n = 8$, $P < 0.05$; Fig. 2b). However, this decrease was abolished in βERKO mice after MPTP treatment (Fig. 2b), suggesting βERKO mice had fewer pain behaviors than WT mice. The hot plate latency of the αERKO mice was similar to the WT mice.

Gene expression of voltage-gated sodium channels in DRG neurons

We analyzed the expression of voltage-gated sodium channels in DRG neurons in ERKO mice after administration of MPTP by quantitative real-time RT-PCR. Our results show that the expression levels of TTX-S (Fig. 3) and TTX-R (Fig. 4) sodium channels were differentially affected in DRGs in MPTP-treated animals. For TTX-S sodium channels, MPTP treatment increased the mRNA expression of Nav1.1 in WT and αERKO ($n = 8$, $P < 0.05$) animals but did not alter its expression in βERKO animals (Fig. 3a). Also, MPTP treatment up-regulated Nav1.7 mRNA expression in WT ($n = 8$, $P < 0.05$), but this increase was lost in both αERKO and βERKO animals (Fig. 3c). We observed that the expression of Nav1.6 significantly decreased in WT and αERKO ($n = 8$, $P < 0.05$) animals but not in βERKO animals (Fig. 3b).

We also explored the expression levels of TTX-R sodium channels in DRGs from MPTP-treated WT, αERKO, and βERKO animals. We observed that MPTP treatment down-regulated Nav1.8 mRNA expression in WT ($n = 8$, $P < 0.05$), but this decrease was lost in both αERKO and βERKO animals (Fig. 4a). The expression levels of Nav1.9 was increased significantly in WT and αERKO ($n = 8$, $P < 0.05$) animals but not in βERKO animals (Fig. 4b).

Discussion

These studies have shown that administration of MPTP (11 mg/kg) to WT mice led to an extensive depletion of

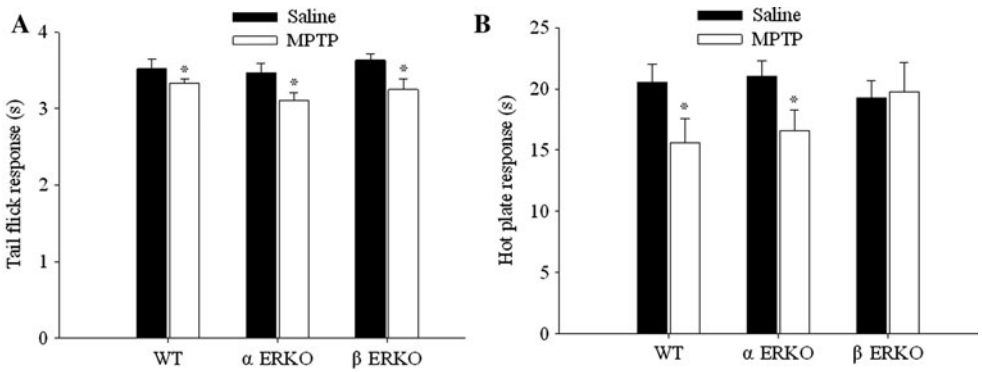

Fig. 2 Nociceptive latencies (s) were measured with tail flick (**a**) and hot plate tests (**b**) 7 days after MPTP compared with controls given saline in the WT and αERKO or βERKO mice. *$P < 0.05$ versus respective intact, saline-treated animals

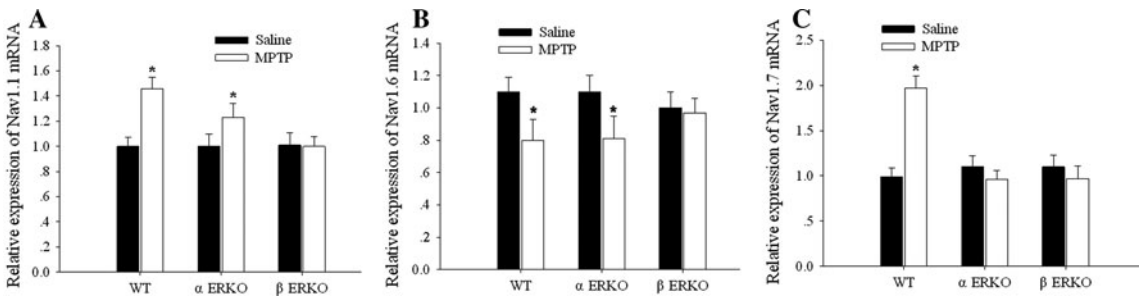

Fig. 3 Alterations of mRNA expression of TTX-S sodium channels in DRG neurons in MPTP-treated animals, dependent on ERs. **a–c** The relative mRNA expression of Nav1.1, Nav1.6, and Nav1.7 in the DRG from saline- and MPTP-treated WT, αERKO, and βERKO C57BL/6 mice ($n = 8$/group). The relative quantity of target mRNA was determined by quantitative real-time RT-PCR using 18s rRNA as an internal control. Each data point represents means ± SEM. *$P < 0.05$ versus respective intact, saline-treated animals

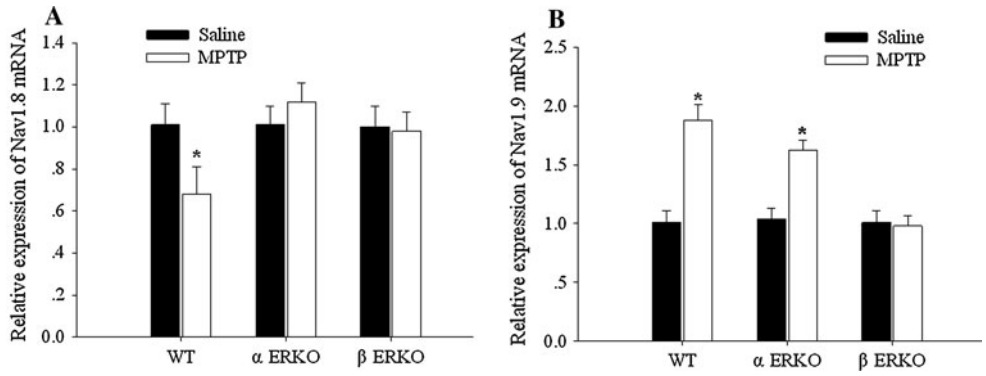

Fig. 4 Alterations of mRNA expression of TTX-R sodium channels in DRG neurons in MPTP-treated animals, dependent on ERs. **a**, **b** The relative mRNA expression of Nav1.8 and Nav1.9 in the DRG from saline- and MPTP-treated WT, αERKO, and βERKO C57BL/6 mice ($n = 8$ per group). The relative quantity of target mRNA were determined by quantitative real-time RT-PCR using 18s rRNA as an internal control. Each data point represents means ± SEM. $^*P < 0.05$ versus respective intact, saline-treated animals

DA and its metabolites DOPAC and HVA, αERKO mice were observed to be more susceptible to MPTP toxicity than βERKO or WT mice. We observed that the injection of MPTP induced a shortened response latency in the tail flick test both in WT and ERKO mice. WT and αERKO mice given MPTP showed a reduced hot plate latency, however, this decrease was abolished in βERKO mice after MPTP treatment. We also found that mRNA expression of TTX-S sodium channels Nav1.1 and Nav1.7 subunits were significantly increased in DRG neurons in MPTP-treated WT animals, and Nav1.6 decreased. The TTX-R sodium channels, Nav1.8, increased in response to MPTP treatment and Nav1.9 decreased.

Pain is an important and distressing symptom in PD. James Parkinson wrote in his famous monograph that painful symptoms can be the first signs of impairment [5]. As Beiske [4] have described, over 80% of patients with PD endured painful symptoms as part of their daily experience with the disorder. Pain is therefore a significant clinical problem in PD and mainly left untreated. However, it is unknown the mechanisms underlying pain in PD. A few studies found that PD patients with or without pain may have low pain threshold [2] and pain tolerance [21]. Defazio and co-workers [22] suggested that pain threshold and pain tolerance tend to decrease as PD progresses, which can predispose to pain development. Consistent with previous results [20], we observed that the injection of MPTP induced a reduced response latency both in the tail flick test and in the hot plate test, indicating that the MPTP-induced lesions of dopaminergic pathways result in hyperalgesia and a decrease in nociceptive thresholds.

Voltage-gated Na^+ channels mediate a rapid and transient increase in Na^+ permeability in response to changes in membrane potential, thereby contributing to the generation and conduction of action potentials that serve as sensory signals from the periphery to the spinal cord through the primary afferent neurons. So the sodium channels in sensory neurons are implicated in the development of inflammatory and neuropathic pain. DRG neurons can express up to five sodium channels more than in any other neuronal cell type [15]. MPTP model is the most widely used PD model for the study of molecular cascades of death of DA neurons [1]. In this study, we found that the expression levels of TTX-S and TTX-R sodium channel subtypes were differentially affected in MPTP model. The mRNA expression of TTX-S sodium channels Nav1.1 and Nav1.7 subunits were significantly increased in DRG neurons in MPTP-treated animals and Nav1.6 decreased. The TTX-R sodium channel, Nav1.8, was increased in response to MPTP treatment and Nav1.9 was decreased. These results support our hypothesis that both TTX-S and TTX-R sodium channels play important roles in the development of pain in PD.

Several studies have reported that estrogens have neuroprotective effects on PD [23–25]. In mice, estrogen pretreatment prevents MPTP-induced depletion of striatal DA. Moreover, estrogen therapy is reported to be beneficial to women with early PD before initiation of L-dopa but not at later stages of the disease. Estrogens improve motor disability in parkinsonian postmenopausal women with motor fluctuations [26]. The neuroprotective effects of estrogen are initiated via ERs. Both ERα and ERβ have been detected in the mouse striatum and substantia nigra. In agreement with previous study [10], we found that administration of MPTP (11 mg/kg) to WT mice led to an extensive depletion of DA and its metabolites DOPAC and HVA, αERKO mice were observed to be more susceptible to MPTP toxicity than βERKO or WT mice.

A substantial body of research indicates that the female patients suffered significantly more from pain than the male patients as in the general population [4] and shows heightened sensitivity to experimentally induced pain compared with men. Regarding activating effects of gonadal hormones, an overwhelming number of studies implicate estrogens as key

modulators of pain in adults [27]. The sex difference in basal mechanical pain threshold and inflammatory hypersensitivity is eliminated in mice lacking either the ERα or ERβ [28]. However, it is unclear which ERs mediate estrogen-related nociception changes. Previous studies have found that estrogens acting primarily through activation of ERβ may also affect spinal cord structures that are important for transmission of nociceptive information [12]. Spooner et al. [29] found that female ERβ knock-out mice showed delayed response in the formalin test compared to WT controls. Furthermore, the selective activation of ERβ by ERb-131 has been shown to provide beneficial effects in animal models of symptoms associated with neuropathic pain [13]. We observed that the injection of MPTP induced a shorter response latency in the hot plate test in the WT and αERKO mice. However, this decrease was abolished in βERKO mice after MPTP treatment, thus we may infer that βERKO mice have stronger pain inhibitory responses than WT mice after MPTP treatment. These results provide evidence for important roles of ERβ in nociception after administration of MPTP, and mechanism of ERs in pain warrant further studies.

The MPTP-induced up-regulation of Nav1.1 and Nav1.9, down-regulation of Nav1.6 in DRG neurons are dependent on ERβ, suggesting that these effects may be through ERβ. We also found that MPTP regulations of Nav1.7 and Nav1.8 are dependent on both ERα and ERβ, suggesting that these effects were through both the receptors. Previous studies have found that external (but not internal) application of 17β-estradiol increased the current amplitude of voltage-gated Na^+ channels in MDA-MB-231 human breast cancer (BCa) cells [30]. In addition, in vivo estradiol significantly decreased the TTX-sensitive sodium currents in isolated gonadotropin-releasing hormone neurons [31].

In conclusion, our study demonstrates the important role of ERs in PD. In DRG neurons, the MPTP-induced up-regulation of Nav1.1 and Nav1.9, down-regulation of Nav1.6 may be through ERβ, whereas the up-regulation of Nav1.7 and down-regulation of Nav1.8 are dependent on both ERα and ERβ. Therefore, the MPTP-induced alterations of gene expression of sodium channels in DRG neurons could be an important mechanism to affect excitability of DRG neurons, and the ERs appear to play a role in nociception in PD.

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Conflict of interest The authors declare that they have no conflict of interest.

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