Programmed Death-1 Deficiency Aggravates Motor Dysfunction In MPTP Model of Parkinson's Disease By Inducing Microglial Activation And Neuroinflammation In Mice

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Research Article

Abstract

Abundant reactive gliosis and neuroinflammation are typical pathogenetic hallmarks of brains in Parkinson's disease (PD) patients, but regulation mechanisms are poorly understood. We are interested in role of programmed death-1 (PD-1) in glial reaction, neuroinflammation and neuronal injury in PD pathogenesis. Using PD mouse model and PD-1 knockout (KO) mice, we designed wild-type-control (WT-CON), WT-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (WT-MPTP), PD-1-KO-control (KO-CON) and PD-1-KO-MPTP (KO-MPTP), and observed motor dysfunction of animal, morphological distribution of PD-1-positive cells, dopaminergic neuronal injury, glial activation and generation of inflammatory cytokines in midbrains by motor behavior detection, immunohistochemistry and western blot. WT-MPTP mouse model exhibited decrease of PD-1/Iba1-positive microglial cells in the substantia nigra compared with WT-CON mice. By comparison of four groups, PD-1 deficiency showed exacerbation in motor dysfunction of animals, decreased expression of TH protein and TH-positive neuronal protrusions. PD-1 deficiency enhanced microglial activation, production of pro-inflammatory cytokines like inducible nitric oxide synthase, tumor necrosis factor-α, interleukin-1β and interleukin-6, and expression and phosphorylation of AKT and ERK1/2 in the substantia nigra of MPTP model. We concluded that PD-1 deficiency could aggravate motor dysfunction of MPTP mouse model by inducing microglial activation and neuroinflammation in midbrains, suggesting that PD-1 signaling abnormality might be possibly involved in PD pathogenesis.

Introduction

Parkinson's disease (PD) is a common and severe neurodegenerative disease in the central nervous system (CNS) with high incidence that is just secondly to Alzheimer's disease (AD). The typical pathological feature of PD is abundant degenerative cell death of dopaminergic neurons in the nigra-striatal system that finally results in dopamine-deficiency in brains and severe motor dysfunction (1−4). Accumulating studies have shown that the neuroinflammation dominated by the activated glial cells, i.e. reactive microglial cells and astrocytes in the CNS, is involved in inducement and progression of dopaminergic neuronal degeneration in PD pathology (5−8). In physiological state, obviously, the microglial cells and astrocytes function in protection of neurons by assisting synaptic transmission, removing dyed cell fragments or foreign harmful molecules, secreting neurotrophic factors and stabilizing microenvironment of CNS neurons (9−11). In pathological conditions, whereas, reactive microglial cells and reactive astrocytes actively join neuroinflammation and neuronal damage, which constitute pathological characteristics of AD, PD, multiple sclerosis, traumatic CNS injury event and other infectious neuropathy (4−8, 12−14). However, detailed significance of inflammatory response and related key regulation factors in modulating glial cell reaction or functional transition from congenital immunity to neuroinflammation state are still unclear (14, 15).

Programmed death receptor-1 (PD-1) is a key checkpoint molecule of immune function, which is a membrane protein expressed in a variety of immune cells such as T-lymphocytes cells, B-lymphocytes, monocytes, natural killer cells, and plays an important roles in regulating antigen response or limiting

host adaptive immune response by threshold of T-lymphocytes and B-lymphocytes, and thereafter joining in prevention of auto-inflammation response or related diseases (16−18). PD-1 ligand PD-L1 and PD-L2 can bind PD-1 receptor, initiate PD-1 signaling and followed biological cellular effects. In tumor microenvironment and inflammatory state, expression of PD-1 in T-lymphocyte increases abnormally. PD-L1 combines PD-1 and initiates a series of inhibition signaling, resulting in decline of T-lymphocyte function, causing immune tolerance of tumor cells and evading T-lymphocyte immune surveillance (19, 20). Evidently, PD-1/PD-L1 signaling pathway is critically in immune function regulation and immune tolerance, thus providing a potential therapeutic way for immune modulatory therapy or intervention of various diseases (21−24).

It is well known that microglial cells belong to the monocyte-macrophage system based on their source of occurrence and characteristics, showing antigen presentation function and secretion of a variety of active factors. PD-L1 is identified in the glial cells and expressional change is observed under CNS inflammatory state (24, 25). However, it is still lack of studies on PD-1/PD-L1 working in the neurodegenerative diseases, especially relation to microglial reaction and neuroinflammatory response. It is interesting to note that a few studies noting PD-1 role in the experimental autoimmune encephalomyelitis and spinal cord injury, and it reveals that PD-1 deficiency results in an increased neuropathological inflammation (26−29). Based on these literature evidences, we speculate that PD-1 signaling pathway may constitute a potential regulating factor in the activated glial cells and inflammation and neuronal damage during pathogenesis of PD. Thus, PD-1 knockout mice and MPTP mouse model were applied for this study (29−33), and immunohistochemistry, western blot and motor behavior detection were used to observe effects of PD-1 deficiency on glial cell reaction, neuroinflammation and motor dysfunction of animals, for purpose to determine possible mechanistic involvement of PD-1 signaling abnormality in PD pathogenesis or progression, which may thereafter provide a new basis for exploring immunotherapy potentially for PD (2, 4).

Materials And Methods

Animals

The wild-type (WT) male C57BL/6 mice (n=30) and PD-1 knockout (KO) male mice (n=20) were used in this study. WT C57BL/6 mice were provided by the Experimental Animal Center of the Fourth Military Medical University. PD-1-KO (*Pdcd-1$^{-/-}$*) mice (a gift form Dr. Tasuku Honjo at Kyoto University, Japan) were produced on C57BL/6 background and introduced into Neurobiology Department of the Fourth Military Medical University (29). Animal experiments were conducted in accordance with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications, eighth edition revised in 2012) and with the approval of Animal Experiment Administration Committee of the Fourth Military Medical University. All efforts were performed to minimize animal number and their suffering during animal manipulations in this study.

MPTP mouse model

Preparation of MPTP mouse model was carried out in reference to a protocol of our previous studies (30–32). Briefly, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Sigma) was intraperitoneally injected at dose of 30 mg/kg/day for consecutive 5 days, and injection of same amount of 0.9% saline was given as control. For detection of cell localization of PD-1 in the substantia nigra and distribution change in MPTP model, we first assigned two group comparison of WT-control and WT-MPTP mice (n=5) at day 5. For assay of effect of PD-1 deficiency on MPTP model, we then assigned four group comparison of WT-control (WT-CON), WT-MPTP (WT-MPTP), PD-1-KO control (KO-CON) and PD-1-KO MPTP (KO-MPTP) mice at time-point of day 5, day 10 and day 15, respectively. The mice in two group comparison were carried out for immunohistochemical staining, and the ones in four group comparison were performed for motor behavior detection, immunohistochemistry and western blot experiment, respectively.

Motor behavior detection

Motor behavior detection were performed for comparison of WT-CON, KO-CON, WT-MPTP, KO-MPTP group at the day 5 (n=10), day 10 (n=5) and day 15(n=5), in which this day-point was labeled since first injection of MPTP solution. For examination of animal active moving and animal balance or grasping function, we setup mouse steel-pole climbing (crawling) and grasping (handholding) experiments. Animal continuing moving time (seconds/per 5minutes) climbing the steel-pole and numbers of falling times (times/per 5 minutes) dropped from the steel-pole were observed and counted in 5 minutes (as count unit). Animal motor dysfunction and changes in different experimental groups of mice were compared and shown.

Immunohistochemistry

Animals under complete anaesthetic state were given perfusion of 4% formaldehyde solution for fixation of brain tissues. The midbrains of mice (n=5) was dissected and placed into 20% sucrose solution for freezing protection of tissues. Freezing sections of midbrains were done at thickness of 15 micron. The midbrain sections containing substantia nigra were rinse in 0.01M PBS buffer for three times and incubated in 0.1% Triton X-100-3% donkey serum for pretreatment of 30 min. Sections were then incubated in required primary antibody solution for 24 hours at 4℃. After 0.01M PBS rinsing, sections were incubated in fluorescence-labeled secondary antibody for 4hours at room temperature. After DAPI counter stain, the sections were observed under a laser scanning confocal microscope (LSCM, Olympus, FX-1000), and interested images were captured for demonstration representatively.

Antibodies and working dilutions were used in immunohistochemical staining. These primary and secondary antibodies were purchased from Sigma, Cell Signaling Technology (CST), DAKO, Wako and Invitrogen. Primary antibodies included rabbit anti-inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-6), arginine-1 (ARG-1), tissue growth factor-β (TGF-β), IL-4, IL-10, AKT, p-AKT, ERK1/2, P-REK1/2 (CST), rabbit anti-Iba1 (Wako), β-actin (Sigma), rat anti-glial fibrillary acid protein (GFAP, DAKO), tyrosine hydroxylase (TH, Sigma), IL-1β, PD-1, PD-L1 (CST) (1:1000 to 1:3000 dilutions, respectively). Second antibodies included Alexa Fluo594-labeled sheep anti-rabbit IgG, Alexa Fluo594-labeled sheep anti-rat IgG, Alexa Fluo488-labeled sheep anti-rabbit IgG, Alexa Fluo488-labeled sheep anti-

rat IgG, HRP-labeled sheep anti-rabbit IgG and HRP-labeled sheep anti-rat IgG (Invitrogen) (1:500 to 1:1000 dilutions, respectively).

Western blot

Western blot was carried out in reference to protocol of our previous study (33). Fresh lysates from midbrains (n=5) were made in lysis buffer (Beyotime Biotechnology). The protein concentrations were measured by BCA, and 10μl (2μg/μl) of sample solution was loaded for each panel. After separation in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (10%), the protein samples were transferred onto PVDF membranes (Millipore). Membranes were then blocked with 5% nonfat milk/TBST (0.1% Tween-20, TBS) for 1hour and incubated with primary antibody respectively at 4°C overnight. The β-actin level was used as an internal control for immunoblot. After incubation with HRP-conjugated secondary antibody at room temperature for 1hour, immunoblots were detected using an advanced ECL detection kit according to manufacturer's instruction. For re-probing, the transfer membrane was stripped by complete washing with stripping buffer (glycine-SDS buffer, pH 2.0) for 15 min, followed by TBST (pH 7.4) three times, and then processed for a standard probing process. Immunoblot images were acquired and analyzed using Bio-Rad's system. Quantification was performed and presented as a density ratio to internal control.

Statistical Analysis

Quantitative data on the immuno-staining cells and immunoblots were shown by Mean ± Standard Error (mean±SEM), and the differences between or among groups were compared by Student T-test (for two groups between WT-CON and WT-MPTP), and One-way ANOVA and Dunnett post-test (for groups among WT-CON, WT-MPTP, KO-CON and KO-MPTP) using GraphPad Prism (5.0) software. When the P-value of comparison between different animal groups was less than 0.05 and considered to have statistical significance.

Results

Morphological distribution of microglial cells showing PD-1 immunoreactivity in the substantia nigra of WT-CON mice

First, double immunofluorescence for PD-1/Iba1, PD-1/GFAP and PD-1/NeuN was carried out to observe localization of PD-1-immunoreactivity in the microglial cells, astrocytes and neurons in midbrains under a laser scanning confocal microscope. PD-1/Iba1 double-labeled microglial cells were observed and numerously distributed in the substantia nigra of WT-CON mice. The PD-1-immunoreactive products were clearly localized in cellular membrane of the microglial cells. On the other hand, PD-1/GFAP double-labeled astrocytes, PD-1/NeuN double-labeled neuronal cells were not observed in the substantia nigra regions of WT-CON mice (Figure 1). In addition, double immunofluorescence for PD-L1/Iba1, PD-L1/GFAP, and PD-L1/NeuN was performed to observe if any PD-L1, a PD-1 ligand, was also localized in microglial

cells, astrocytes and neurons, and data indicated that PD-L1 was mainly located in the microglial cells rather than astrocytes and neurons (data not shown).

Significant decrease of microglial cells with PD-1/Iba1 double-labeling in the substantia nigra of WT-MPTP mice

Then, immunohistochemstry was again applied to observe if any changes of PD-1/Iba1 double-labeled microglial cells distributed in the substantia nigra occurred in MPTP model. Both Iba1-immunopositive microglial cells and GFAP-positive astrocytes in MPTP mice showed active morphology with an increase in their numbers, which was accompanied with dopaminergic neuronal death in the substantia nigra and basically consistent with results of previous studies (3, 5, 30−32). Compared with WT-CON group, the microglial cells with PD-1/Ibal double-positivity decreased significantly in their numbers in WT-MPTP mice, and immunostaining intensity of PD-1 was also weakened in WT-MPTP mice. Comparison of corresponding nigral regions between WT-CON and WT-MPTP group showed differences in morphology and numbers of PD-1/Ibal double-positive cells (Figure 2). Cell count on PD-1/Iba1 double-positive microglial cells in unit area of one-sided nigral sections indicated that 280±32 in WT-CON group and 189±26 in WT-MPTP group, and statistical comparison showed significant differences between these two groups (*Student T-test*, *P*<0.01).

Aggravating effect of PD-1-deficiency on animal motor dysfunction and reduction of TH-positive protrusions in KO-MPTP mice

After that, PD-1-knockout mice was applied to assess effect of PD-1-deficiency on the motor function and survival of dopamine neurons in MPTP model by comparing animal movement function (moving time and falling times) and immunohistochemical visualization of TH nigral neurons at the d5, d10, d15 among WT-CON, WT-MPTP, KO-CON and KO-MPTP group. Compared with WT-MPTP, motor dysfunction in KO-MPTP mice was more severe, moving performance was slower, moving time was shortened, the falling times increased obviously, or animals were unwilling to move or even stay in place. The WT-CON mice also showed a decrease in moving time compared to KO-CON group, but there was no significant change in falling times (Figure 3). Immunohistochemistry and western blot were further done to show TH-positive neurons, neuronal protrusions and TH expression levels. Numbers of TH-positive neurons in WT-MPTP group decreased significantly compared with WT-CON mice, which was consistent with our previous observations (30−32). Compared with WT-MPTP, there was no significant difference of TH-positive neurons in their numbers of in the KO-MPTP mice. But, KO-MPTP mice showed decrease of TH-positive neuronal protrusions or cell processes compared with that of WT-MPTP group (*P*<0.05) (Figure 4A, B). Immunoblot data also confirmed that KO-MPTP mice showed decrease in expression level of TH protein compared with that of WT-MPTP group (*P*<0.01) (Figure 4C).

Promoting effects of PD-1-definciency on the microglial activation and expression levels of proinflammatory cytokines in KO-MPTP mice

Furthermore, effects of PD-1 knockout state on the glial cell activation, expression levels of proinflammatory factors and anti-inflammatory factors were analyzed to evaluate PD-1 participation in neuroinflammatory response. The KO-MPTP mice had a significant increase of Iba1 expression level compared with WT-MPTP group (P<0.01), while expression of PD-1, PD-L1 and GFAP did not show any significant changes (P>0.05) (Figure 5). Expressions of proinflammatory cytokine iNOS, TNF-α, IL-1β and IL-6 showed up-regulated trend in both KO-CON and KO-MPTP group. The KO-MPTP mice increased expression of iNOS, TNF-α, IL-1β and IL-6 significantly compared to WT-MPTP group. The KO-CON group also showed upward changes of iNOS, IL-1beta, and IL6 except of TNF-α compared to WT-CON group (Figure 6). Double immunostaining showed positive localization of iNOS, TNF-α, IL-1β and IL-6 in the microglial cells (data not shown). In addition, anti-inflammatory cytokine ARG-1, TGF-β, IL-4, IL-10 expression change trend was not fully consistent. Compared with WT-MPTP group, ARG-1 showed a significant downward change, TGF-β and IL-4 no significant change, while IL-10 showed a clear upward trend in expression in KO-MPTP mice (Figure 7). It indicated that PD-1-deficiency increased microglial activation and production of proinflammatory cytokines, and it might thereafter induce or aggravate neuroinflammatory response, dopaminergic neuronal damage and motor dysfunction in KO-MPTP mice.

Motivating influence of PD-1-definciency on AKT and ERK1/2 signaling pathway in KO-MPTP mice

Finally, western blot was applied to detect possible signaling mechanism of PD-1-deficiency affecting glial activation and inflammatory response by examining AKT, p-AKT, ERK1/2 and p-ERK1/2 expression in the substantia nigra of in above four animal groups. Compared with WT-MPTP group, expression levels of p-AKT, tAKT, p-ERK1/2 and tERK1/2 increased significantly in KO-MPTP mice (Figure 8). At the same time, ratios of p-AKT to tAKT, or p-ERK1/2 to tERK1/2 also increased in KO-MPTP group. Data showed that phosphorylation or activation of AKT and ERK1/2 signaling increased to a certain extent in KO-MPTP group, indicating that PD-1-deficiency possibly promoted activation of AKT and ERK1/2 signaling pathway in KO-MPTP mice. However, whether AKT, p-AKT, ERK1/2, p-ERK1/2 signaling molecules were exactly or mainly localized in the activated microglial cells or astrocytes was subject to further experimental confirmation.

Discussion

In this study, PD-1 knockout mice and MPTP model of Parkinson's disease (PD) were used to observe effects of PD-1-deficency on motor dysfunction of animals, glial cell reaction, and neuroinflammation in the substantia nigra. It revealed that PD-1/Iba1 double-immunopositive microglias reduced in WT-MPTP mice compared with that of WT-CON ones. Compared with WT-MPTP group, KO-MPTP mice showed increases in the motor dysfunction, decreased expression of TH protein and TH-positive neuronal protrusions, which was accompanied by microglial and astroglial activation, increased expression of proinflammatory cytokine iNOS, TNF-α, IL-1β and IL-6. Further signaling detection showed that PD-1-KO mice induced elevated expression and phosphorylation activation of AKT and ERK1/2. Results of this

study together indicated that PD-1 deficiency could aggravate motor dysfunction of PD animal model, possibly by promoting microglial cell reaction, increasing proinflammatory cytokine generation and triggering AKT and ERK1/2 signaling activation in the midbrains, suggesting that PD-1 signaling abnormality might be most possibly involved in microglial activation and neuroinflammation in PD pathogenesis and progression.

Many studies showed that abnormal activation of microglial cells and astrocytes in the CNS dominated neuroinflammatory reaction, which might be a critical factor leading to the development of various neurodegenerative diseases such as AD, MS and PD (4–8). In the state of inflammatory reaction, microglial cells functioned as CNS inherent immune cells, activated microglial cells initiated differentiation in two opposite directions, namely, inflammatory (M1 state), anti-inflammatory (M2 state) functional polarization (29). On the one hand, microglial cells in M2 polarization could remove pathogens or cell fragments and protect from damage to the brains. On the other hand, microglial cell with M1 polarization was in an inflammatory state and could produce a series of cytokines, and thereby further activating astrocytes, being collaborative inflammatory response. The astrocytes regulated the immune response and react to pathological changes by hypertrophy, presented as functional activated state, the activated astrocytes also occurred in similar functional differentiation, that is, inflammatory state (A1) and anti-inflammatory state (A2) polarization, activated A2 astrocytes promoted tissue repair and help maintain function of the central neurons. The activated A1 astrocytes secreted a large number reactive oxygen and pro-inflammatory cytokines, affecting neurons and other glial function, triggering a vicious circle, exacerbating the biological process of amplification of inflammatory reactions and neuronal damage in the CNS (34, 35).

It was known that as an important inhibitory immune checkpoint, PD-1 is the regulating molecule of immune cell function (36). Studies showed that PD-1 and PD-L1 expression levels changes along with healthy or pathological states in the CNS, for example, 20% of microglial cells expressed PD-L1 in uninfected healthy mice, while more than 90% of microglial cells showed induced PD-L1 expression 1week after infection (24, 25). Stimulation of interferon-gama (IFN-γ), autoimmune diseases, brain tumors and stroke state induced central T-cell activation and PD-1 production. Increased PD-L1 binding and activation of PD-1 signaling pathway regulated tumor microenvironment and inflammatory response, and thus affected progression of above diseases (37). Some studies indicated that PD1/PD-L1 signaling activation promoted differentiation of microglial cells into anti-inflammatory states (M2) and reduced secondary brain damage in the cerebral hemorrhage (38, 39). In addition, many studies focusing on brain tumors showed that tumor cells secreted high level of PD-L1, which thereafter induced T-lymphocytes to produce high level of PD-1 molecule and exact biological effects. Combination of PD-1/PD-L1 or PD-1 signaling activation caused decrease or "failure" state of T-cell function, resulting in the migration and diffusion of tumor cells. As a targeted anti-tumor strategy, therefore, PD-1-based inhibitors (PD1 antibody) have attracted more and more attention and successfully used in clinical.

Whether PD-1 signaling pathway was involved in glial cell activation and inflammation of PD, which in turn affected course of disease, was major concerning question of this study. Present results showed that

PD-1 deficiency state leaded to more obvious glial activation in MPTP mouse model, increased the expression level of inflammatory factors, and aggravated motor dysfunction of animals, which indicated that PD-1 had a certain restrictive effect on neuroinflammation dominated by activated glial cells, and played a neuroprotective role by regulating or limiting inflammatory response. Our result was supported by studies of Yao and other researchers. Yao and other applications used PD-1 knockout and spinal cord injury model, reveal that PD-1 knockout promoted direction of microglial cells and macrophage M1 polarization, and aggravated inflammatory response and neuronal damage (29). Contrary to these observations, Bodhankar used stroke models and found that PD-L1 deficiency improved infarction volume, reduced inflamed cells and inflammatory responses, and improved nerve function (40). It was not clear what causes difference of studies, which might be related to differences in application of animal models. Other scholars also reported that PD-1 signaling showed a protective role in persistent viral encephalitis. PD-1 signaling activation limited severity of inflammation during acute infection while it maintained a moderate inflammatory response during persistent infection and conduced to resistance to viral re-infection (41, 42). It remained a question whether of PD-1 signaling activation might produce a "double-edged sword" effects in different CNS diseases or distinct stages of disease. It was also evidenced that PD-1 showed a protective role by limiting inflammatory strength through glial inhibition of T-lymphocyte function (43). It was notice that, in addition, we still detected positive-band of PD-1 by western blot, indicating that PD-1 antibody showed cross-reaction in PD-1 KO mice used, and PD-1-KO information showed knockout of extracellular domains in this strain of PD-1 KO mice and this might only make PD-1 molecule lose functional binding of ligands instead of whole deletion of PD-1 expression. Nevertheless, further studies on mechanism of PD-1/PD-L1 signaling in abundant glial reaction and neuroinflammation in the CNS shall be helpfully expected to identify new intervention targets for manipulation of neuroinflammatory balance and to develop new strategies against PD pathogenesis and progression (2, 4, 44).

Conclusions

By using PD-1-KO mice and MPTP mouse model, this study revealed that PD-1-deficency aggravated motor dysfunction of PD animals by promoting the microglial cell activation and enhanced neuroinflammatory reaction in the substantia nigra. This study further suggested that PD-1 signaling abnormality might be possibly involved in PD pathogenesis or progression.

Abbreviations

AD, Alzheimer's disease; ARG1, arginase 1; GFAP: glial fibrillary acidic protein; IFNγ: interferon-γ; IL-6, interleukin-6; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PD-1, Programmed death receptor-1; PD-L1, Programmed death ligand 1; TGF-α, transforming growth factor-beta; TNF-α: tumor necrosis factor-alpha.

Declarations

Authors' contributions

Ying-Ying Cheng, Bei-Yu Chen: Methodology, Writing-original draft preparation.

Gan-Lan Bian: Formal analysis and Validation.

Liang-Wei Chen, Yin-Xiu Ding: Conceptualization, Writing-review & editing.

Availability of data and materials

Data and materials from this manuscript are available upon request.

Ethics approval and consent to participate

Animal experiments were conducted in accordance with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications, eighth edition revised in 2012) and under the approval of the Animal Experiment Administration Committee of the Fourth Military Medical University.

Consent for publication

All authors have read the manuscript and approved submission for publication.

Competing interests

The authors declare that they have no competing interests.

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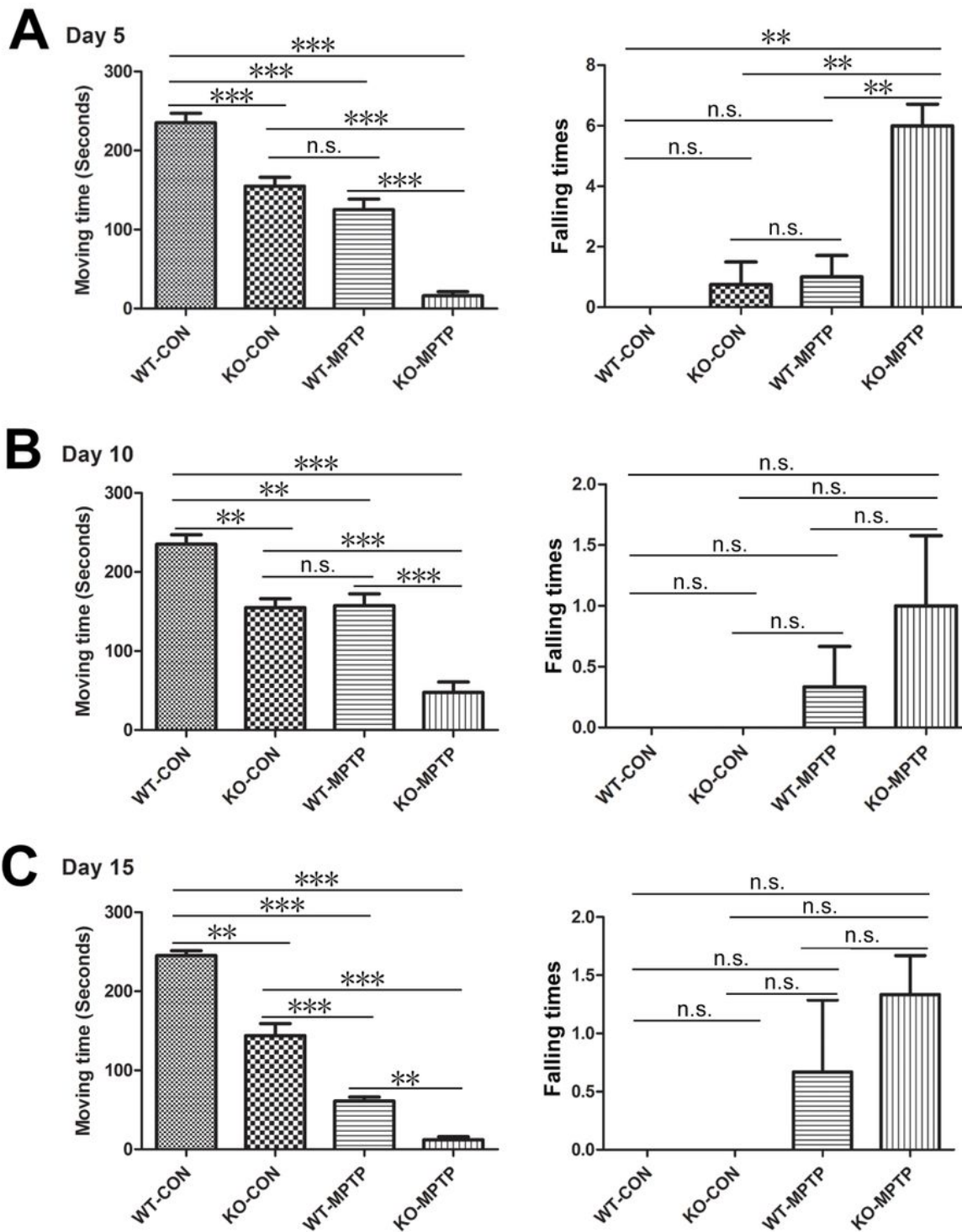
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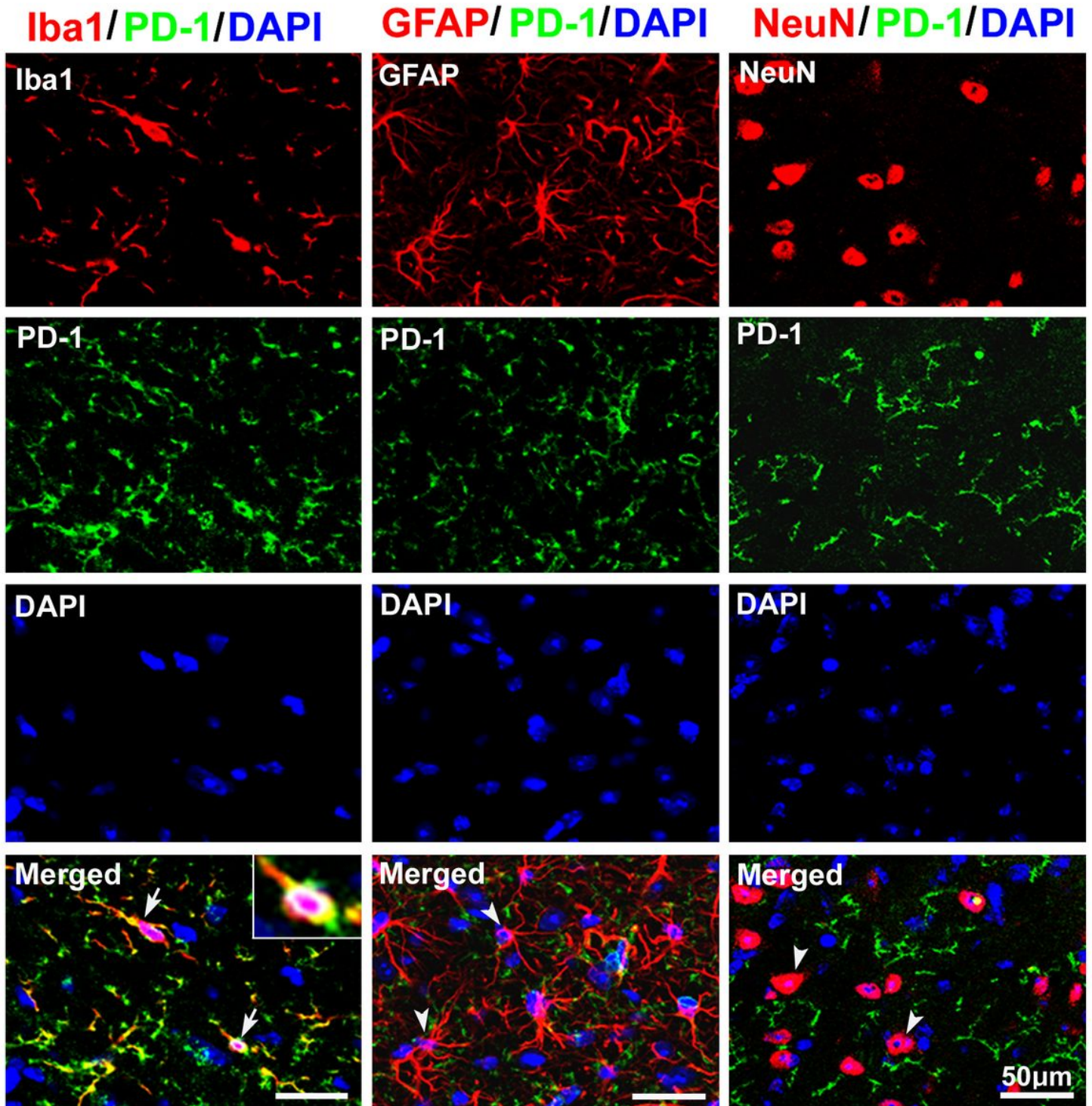
Figure 1

Double immunofluorescence showing PD-1/Iba1, PD-1/GFAP, PD-1/NeuN-positive cells. Distribution of PD-1/Iba1 double-positive microglial cells (arrows), GFAP-positive astrocytes (arrowheads), NeuN-positive neurons (arrowheads) are seen in the substantia nigra of WT-CON mice.
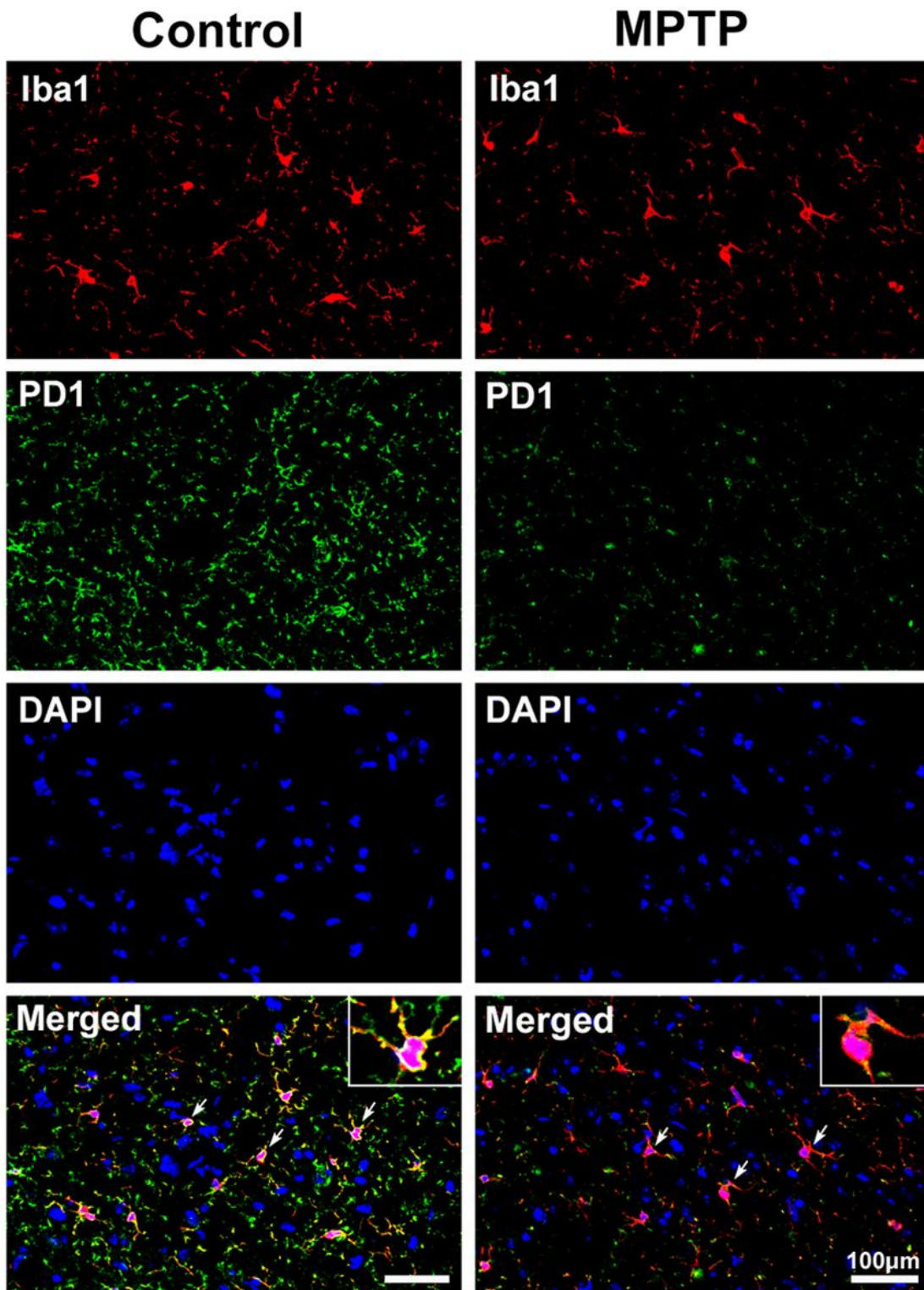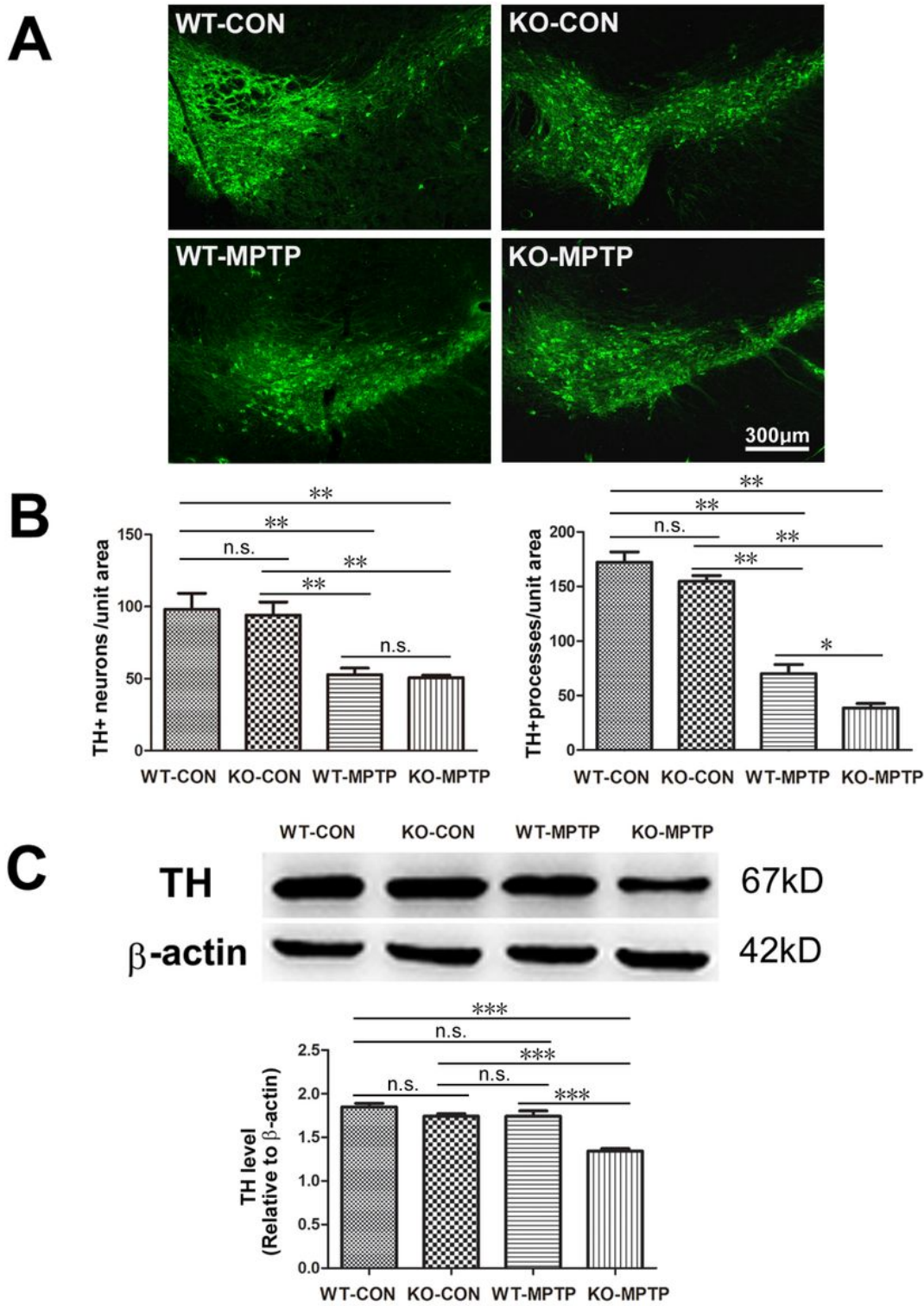
Figure 2

Morphological distribution of PD-1/Iba1 double-positive microglial cells in the WT-CON and WT-MPTP group. PD-1/Iba1 double-positive microglial cells are numerously in the substantia nigra of WT-CON and decrease in WT-MPTP mice. PD-1 immunostaining intensity decreases and Iba1-positive cell process becomes shorter in WT-MPTP mice.

Figure 3

Motion behavior detection shows moving time (climbing ability) and falling times (handholding or grasping ability) during 5 minutes in WT-CON, KO-CON, WT-MPTP and KO-MPTP group. A-C, Moving time (seconds) and falling times (numbers) in d5, d10 and d15 group, respectively. ANOVA: **P<0.01, *** P<0.001, n.s. no significance (n=10).
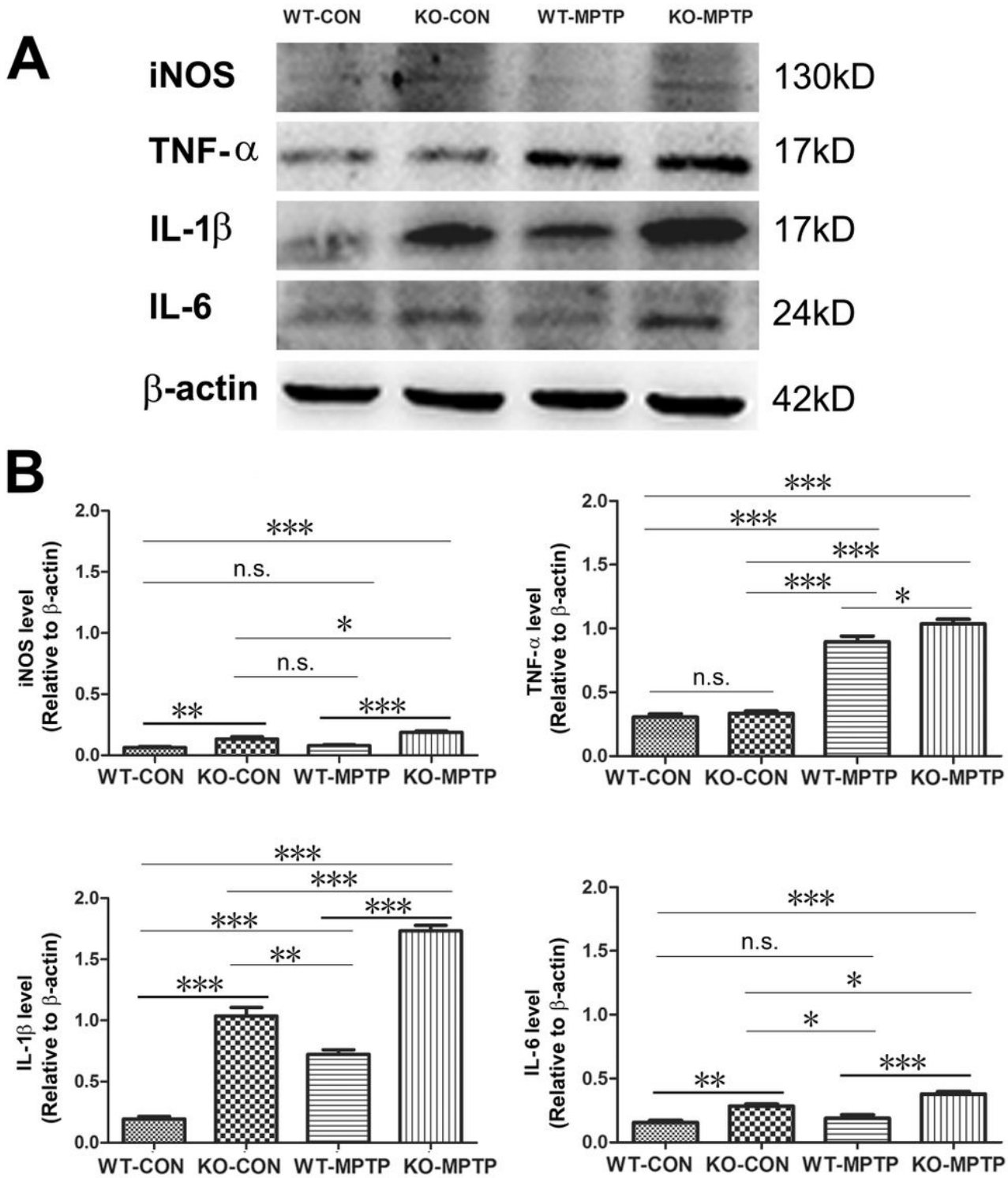
Figure 4

Distribution, quantity of TH-positive dopamine neurons and TH expressional levels in WT-CON, KO-CON, WT-MPTP and KO-MPTP group. A, TH-positive dopaminergic neurons in the substantia nigra; B, Data comparison of TH-positive neuronal bodies and protrusions; C, Immunoblots of TH expression and comparison of levels among groups. ANOVA: *P<0.05, **P<0.01, ***P<0.001, n.s. no significance (n=5).
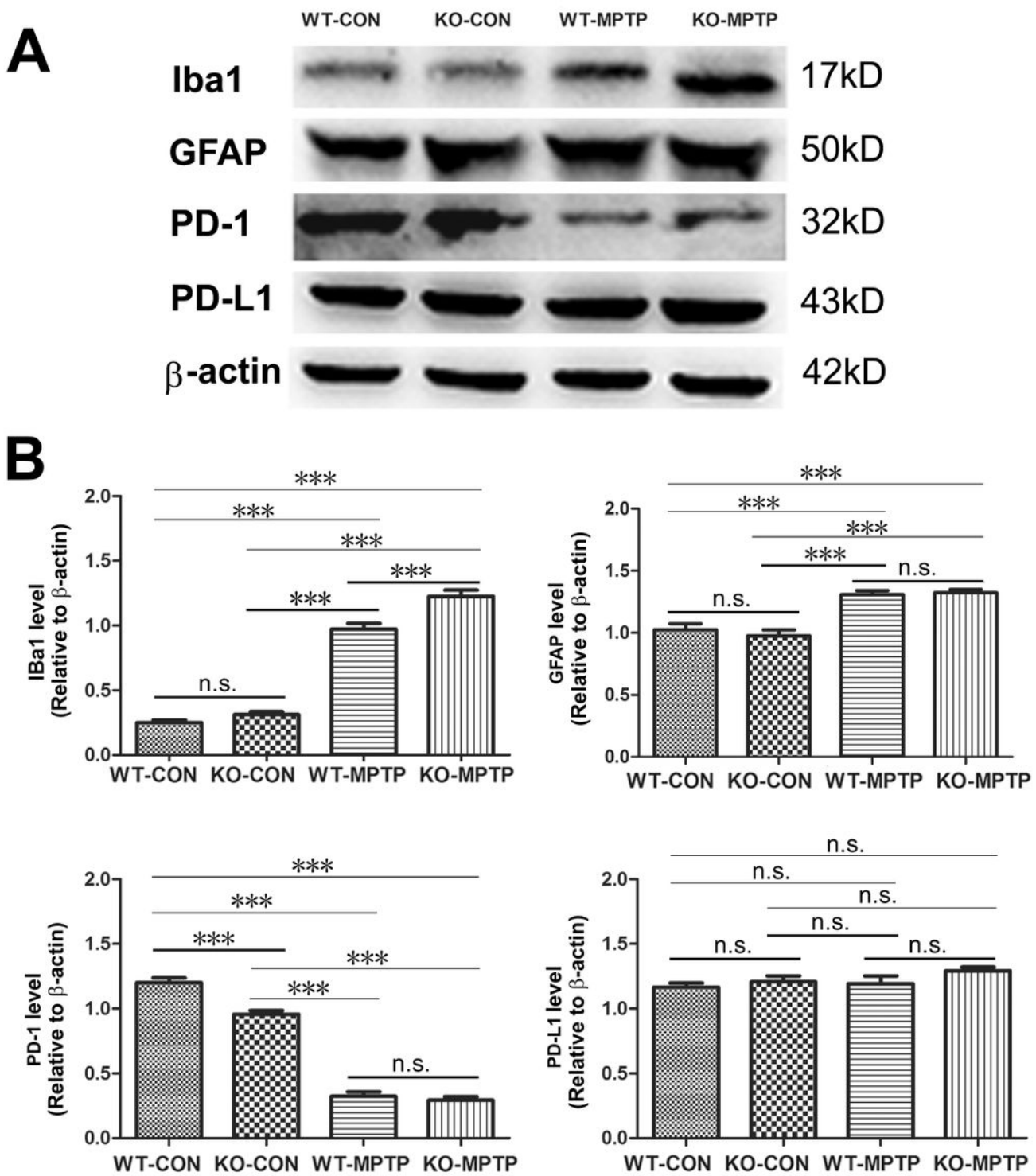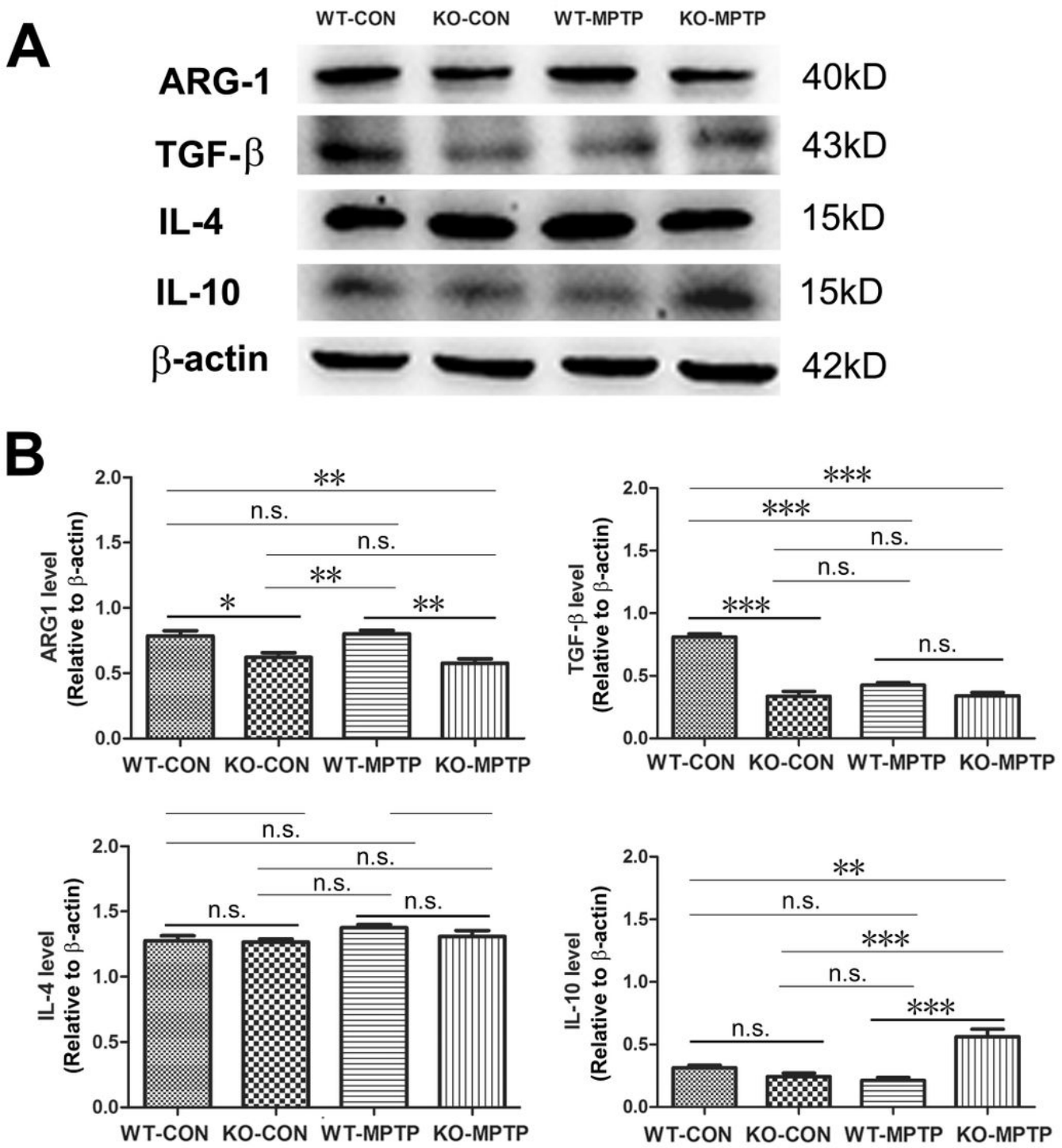
Figure 5

Expression and changes of iba1, GFAP, PD1 and PD-L1 in WT-CON, KO-CON, WT-MPTP and KO-MPTP group. A, Immunoblots of iba1, GFAP, PD-1 and PD-L1; B. Comparison of iba1, GFAP, PD1 and PD-L1 levels among groups. ANOVA: ***P<0.001, n.s. no significance (n=5).

Figure 6

Expression of pro-inflammatory cytokine iNOS, TNF-α, IL-1β and IL6 in WT-CON, KO-CON, WT-MPTP and KO-MPTP group. A, Immunoblots of iNOS, TNF-α, IL-1β and IL6; B. Comparison of iNOS, TNF-α, IL-1β and IL6 level among groups. ANOVA: *P<0.05, **P<0.01, ***P<0.001, n.s. no significance (n=5).

Figure 7

Expression of anti-inflammatory cytokine ARG1, TGF-β, IL4 and IL10 in WT-CON, KO-CON, WT-MPTP and KO-MPTP group. A, Immunoblots of ARG1, TGF-β, IL4 and IL10; B. Comparison of ARG1, TGF-β, IL4 and IL10 level among groups. ANOV: *P<0.05, **P<0.01, ***P<0.001, n.s. no significance (n=5).
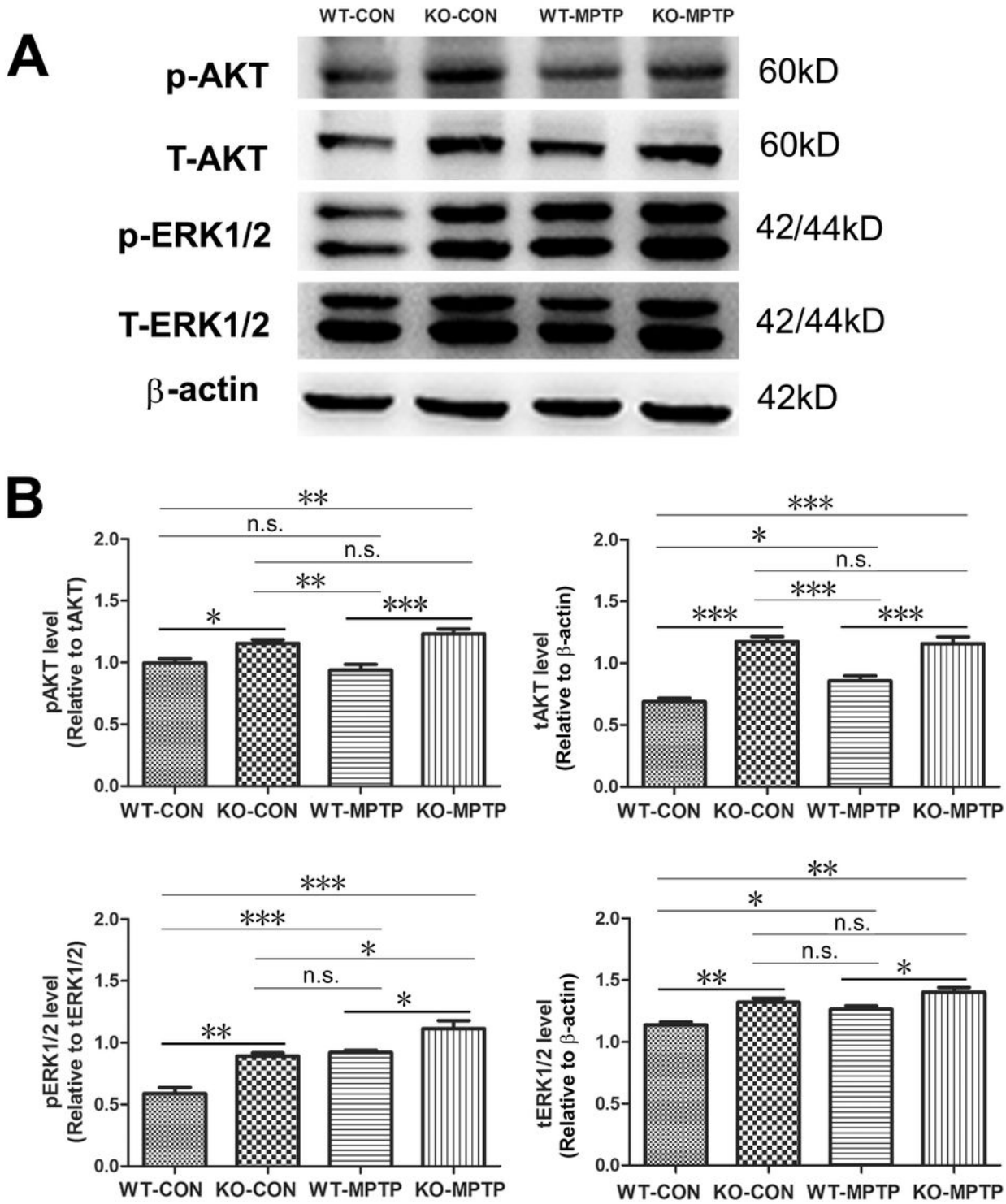
Figure 8

Expressional levels of p-AKT, tAKT, p-ERK1/2 and tERK1/2 in WT-CON, KO-CON, WT-MPTP and KO-MPTP group. A, Immunoblots of p-AKT, tAKT, p-ERK1/2 and tERK1/2; B. Comparison of p-AKT, tAKT, p-ERK1/2 and tERK1/2 level among groups. ANOVA: *P<0.05, **P<0.01, ***P<0.001, n.s. no significance (n=5).