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Meloxicam ameliorates motor dysfunction and dopaminergic neurodegeneration by maintaining Akt-signaling in a mouse Parkinson's disease model

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ABSTRACT

A series of oxicam non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be neuroprotective against 1-methyl-4-phenyl pyridinium in human neuroblastoma SH-SY5Y cells via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway independent of cyclooxygenase (COX) inhibition. The present study endeavored to establish this novel effect of meloxicam (MLX), an oxicam NSAID, in a mouse Parkinson's disease (PD) model using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Male C57BL/6 mice, which received MPTP (30 mg/kg/day; s.c.) for 5 consecutive days (chronic model) with 10-day follow-up saline administrations, showed significant motor dysfunction in the pole test due to reduced tyrosine hydroxylase (TH) protein levels in the brain on day 16 after MPTP/saline treatment. Daily coadministrations of MLX (10 mg/kg/day; i.p.) and MPTP for the first 5 days and follow-up 10 days with MLX administrations alone (MPTP/MLX treatment) significantly ameliorated MPTP-induced behavioral abnormalities in mice. Concomitant decreases of TH protein levels in the striatum and midbrain of MPTP/MLX-treated mice were not only significantly ($p<0.01$ and $p<0.05$, respectively) ameliorated but phosphorylated Akt (pAkt473) expression in the midbrain was also significantly ($p<0.01$) increased in the midbrain when compared with MPTP/saline-treated mice. These results suggest that MLX, an oxicam NSAID, attenuated dopaminergic neuronal death in the experimental MPTP-PD model by maintenance of the Akt-signaling. Oxicam NSAIDs may serve as potential drugs for PD treatment via a novel mechanism of action.

Keywords: Oxicam; non-steroidal anti-inflammatory drugs (NSAIDs); Neuroprotection;

Parkinson's disease (PD); Akt; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

1. Introduction

Parkinson's disease (PD) is characterized by the progressive dopaminergic neurodegeneration in the substantia nigra pars compacta of the brain [6,14]. Although much-coveted, disease-modifying drugs for PD treatment via the prevention of neuronal death in the PD brain are not available as yet [8,17]. The Akt pathway, or prosurvival intracellular signaling, is markedly deactivated in the substantia nigra pars compacta of PD patients [10,26]. Therefore, activating the Akt pathway may serve as one of the most promising targets for effective PD treatment [4,19].

Two oxicam non-steroidal anti-inflammatory drugs (NSAIDs), meloxicam (MLX) and piroxicam, have been reported to attenuate dopaminergic neurodegeneration induced by a single-bolus administration (30 mg/kg, i.p.) of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in vivo and in vitro [20,24]. Accordingly, it has been concluded that cyclooxygenase§ (COX) inhibition is responsible for attenuating MPTP toxicity via a mechanism of neuroprotective action in the affected brain regions. In fact, acute treatment of MPTP (20 mg/kg/day x 4 days; i.p.) induces microglial activation in the mouse brain [3]. However, in a chronic MPTP-PD model using C57BL mice (30 mg/kg/day for 5 consecutive days), inflammatory responses have not been clearly observed in the substantia nigra and striatum [3]. On the other hand we have recently demonstrated that oxicam NSAIDs, but not other categories of NSAIDs, elicit a neuroprotective action against MPP^+-induced SH-SY5Y neuroblastoma cell death [22,23] via maintenance of phosphatidylinositol 3-kinase (PI3K)/Akt-signaling, a pathway which is independent of COX inhibition. Moreover, it has been indicated that activation of the

PI3K/Akt-signaling is known to be neuroprotective in PD-related cell lines and animal models [1,11,18].

In this study, we investigated whether MLX attenuated or reversed neurodegeneration and behavior abnormality in chronically MPTP-treated mice, and probed further if the effective outcome was established via the involvement of PI3K/Akt-signaling.

2. Materials and methods

2.1. Materials

MLX sodium hydrate (MLX), and MPTP hydrochloride (Sigma; St Louis, MO, USA), anti-tyrosine hydroxylase (TH) antibody (Millipore; Billerica, MA, USA) and other primary antibodies (Cell Signaling Technology; Danvers, MA, USA) were commercially acquired. In addition, horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies from sheep and donkey (GE Healthcare; Little Chalfont, UK) were used, respectively. All other chemicals (Wako; Osaka, Japan) were of reagent grade. Drugs were all dissolved in physiological saline, and saline was therefore used as the vehicle.

2.2. Animal model with chronic MPTP treatment

Male C57BL/6 mice weighing 20-23 g (7-8 weeks old; SLC, Shizuoka, Japan) were accommodated in a temperature-controlled room (4-6 mice/cage) under alternating 12-h light/dark cycle with water and pellet food given ad libitum. After 1-week acclimatization,

mice were injected either with saline (i.p.) and saline (s.c.) (i.e. control); saline (i.p.) and MPTP (30 mg/kg/day, s.c.) per se (i.e. MPTP group); or MLX (10 mg/kg/day, i.p.) and MPTP (30 mg/kg/day, s.c.) (i.e. MPTP/MLX group) for the first 5 consecutive days. Note that: all administrations were performed at 10 ml/kg in all groups. From day 6 after the initial treatment, the control, MPTP and MPTP/MLX groups were followed-up daily with saline (i.p.), saline (i.p.) and MLX (10 mg/kg, i.p.) until day 15 (i.e. 10-day follow-up treatment), respectively. The mice were euthanized on day 16 after the initial vehicle/agent treatment with pentobarbital, and the striatum and midbrain (without hypothalamus) were isolated from the murine brain and stored at -80°C until use. This animal experiment was approved by the Ethical Committee for Appropriate Animal Experiments at Asahikawa Medical University, and was performed in compliance with the Japanese Experimental Animal Guidelines.

2.3. Evaluation of behavioral abnormalities

On day 15 after the initial vehicle/agent administration, or on the day before the last MLX treatment, the pole test (with slight modification) was conducted for evaluation of behavior abnormalities [16,21]. Briefly, a mouse was grasped with its head upright and placed on top of a 55-cm-high pole (diameter: 8 mm) wrapped with gauze. The time-intervals taken from the beginning of its movement to having with its head completely turned downward (Tturn), and from the beginning to landing completely on the floor (TLA) were monitored. Each mouse was given four trials for measurement of the respective time-intervals.

2.4. Western blotting

Brain tissues were homogenized and lysed using TissueLyser II (Qiagen, Hilden, Germany) in 200-500 μl of ice-cold lysis buffer containing 20 mM Tris buffer (pH 7.5), 250 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 10 mM NaF, 2 mM sodium orthovanadate and the protease-inhibitor cocktail (EDTA-free complete type; Roche Applied Science, Switzerland). Tissue debris was removed from the lysate by centrifugation (15,000 x g, 10 min) at 4°C, and the protein contents of supernatant were determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). After boiling with Laemmli buffer for 5 min, proteins (15 or 25 μg) separated by 8.5% SDS-polyacrylamide gel electrophoresis were electrophoretically transferred onto a polyvinylidene fluoride membrane. The membrane was then sequentially treated with Block Ace (**DS Pharma** Biomedical, Osaka, Japan) and incubated overnight at 4°C with each first antibody such as antiphosphorylated-Akt (Ser 473; 1:1,000) from rabbit, anti-Akt (1:10,000) from rabbit or anti-TH (1:30,000) from mouse in TBST (10 mM pH 7.5 Tris buffer containing 0.9% NaCl, 0.05% Tween 20 and 10% methanol), respectively. For rabbit β-actin antibody, a dilution rate of 1:1,500 in TBST was used. The membrane was then washed three times with fresh TBST and probed with the HRP-conjugated anti-rabbit or mouse IgG antibody from donkey (1:10,000) for 1 h at room temperature. The washing procedure was repeated three times before the membrane was treated with a chemiluminescent reagent (ECLplus; GE Healthcare, Little Chalfont, UK). Proteins were visualized using an LAS3000 image-analyzer (FUJIFILM, Tokyo, Japan).

2.5. Statistical Analysis

Data were analyzed by one factorial ANOVA followed by the Bonferroni multiple comparison test for the post-hoc significance testing. Differences where $p<0.05$ were considered statistically significant.

3. Results

3.1. Effect of MLX on bradykinesia induced by chronic MPTP injections.

Motor dysfunction (bradykinesia) was indexed according to the time-lengths of Tturn and TLA in the pole test (Fig. 1). MPTP-treated mice showed significantly ($p<0.001$) longer intervals in Tturn and TLA than those of non-treated control mice. However, MPTP-induced bradykinesia (both monitored according to Tturn and TLA) were significantly ($p<0.001$) reversed with MPTP/MLX coadministration to control levels (Fig. 1).

3.2. Effect of MLX on reduced protein levels of TH in the striatum and midbrain of chronically MPTP-treated mice

TH contents were measured as a marker for the respective numbers of dopaminergic terminals and cell bodies in the striatum and midbrain. The TH level in the

striatum was markedly ($p<0.001$) reduced (13.9% of control) by chronic MPTP administration (Fig. 2A); however, this decrease was significantly ($p<0.01$) reversed in mice treated with MPTP/MLX coadministration (27.0% of control). In a similar tendency, although MPTP treatment significantly ($p<0.001$) reduced TH levels in the midbrain to 66.7% of control, this decrease in the affected mice significantly ($p<0.05$) recovered to 84.7% of control after MPTP/MLX coadministration (Fig. 2B). The β-actin levels in the striatum and midbrain were not affected between any two groups.

3.3 Effect of MLX on reduced protein levels of phosphorylated Akt in the midbrain of chronically MPTP-treated mice

We investigated the effect of a single subcutaneous administration of MPTP (30 mg/kg/day) on pAkt (Ser 473) levels in the murine midbrain in our preliminary study. The pAkt levels in the midbrain were not altered when compared with those of saline-treated mice after a single-injection with MPTP (data not shown). However, pAkt levels in the midbrain were significantly ($p<0.05$) reduced after chronic MPTP treatment (Fig. 3A). In addition, MPTP/MLX co-administration significantly prevented MPTP-induced pAkt reduction (Fig. 3). Interestingly, the total Akt levels were not different among the three groups (Fig. 3B): i.e. single MPTP injection, chronic MPTP treatment, and MPTP/MLX co-administration. In addition, any significant difference in the ratio of pAkt(473) to total Akt was not observed between the control and MPTP/MLX co-administration groups (Fig. 3C).

4. Discussion

Chronic treatment of MPTP induced motor dysfunction accompanied with a significant reduction in TH protein contents in the striatum and midbrain of C57BL/6 mice. These results are most likely due to dopaminergic neural degeneration induced by MPTP [1,7,15]. In this study, we demonstrated that MLX, an oxicam NSAID, significantly ameliorated MPTP-induced dopaminergic neurodegeneration evaluated by the pole test and TH content. The most important finding was that this neuroprotective effect of MLX was accompanied by the maintenance of Akt-signaling.

Repeated treatment of MPTP on a daily basis has been shown to induce injury of dopaminergic neurons in C57BL mice via the mitochondrial apoptotic cascade: i.e. a complex constellation consisting of Apaf-1, cytochrom c and caspase-9 [13]. The involvement of this cascade has been believed to be a main feature in PD etiology [12]. On the other hand, the inflammatory pathway via microglial activation has been preferentially observed in the acute MPTP-PD model that employs four MPTP (20 mg/kg; i.p.) injections at 2-h intervals [11,12]. Indeed, oxicam-nonbearing NSAIDs, such as indomethacin and rofecoxib, elicit their neuroprotective action in the acute MPTP-PD model via COX inhibition [9,25]. On the contrary, this is the first study to demonstrate that NSAIDs displayed neuroprotective effects in the chronic MPTP-PD mouse model. In a cellular model using neuroblastoma SH-SY5Y in our previous studies [22,23], only oxicam-bearing NSAIDs exhibit neuroprotective effect against MPP^+-induced apoptosis via the anti-apoptotic cascade and PI3K/Akt pathway independent of COX inhibition. The findings in a cellular model [22,23] are strongly supported by the present results using the chronic

MPTP-PD mouse model, although oxicam-nonbearing NSAIDs were not evaluated in this study.

Accordingly, the current neuroprotective effect of MLX (an oxicam NSAID) against chronic MPTP treatment was probably attributable to prevention of pAkt reduction at Ser473 by repeated MLX administration. This down-regulation of pAkt by MPTP in the murine midbrain has also been documented in previous studies [2,18]; viz., Akt phosphorylation at Ser473 and Ser308 decreased 12 h after a single-bolus administration of MPTP (30 mg/kg) in mice. However, our preliminary data in this study showed that the Akt phosphorylation at Ser473 was not affected 7 days after a single-bolus administration of MPTP. Taken together, Akt phosphorylation was probably transiently down-regulated, and it recovered to normal levels within 7 days after a single-bolus administration of MPTP. On the contrary, daily MPTP administrations may continuously down-regulate Akt phosphorylation to eventually induce apoptotic cell death. Thus, the neuroprotective mechanism of MLX could be mediated by maintaining PI3K/Akt-signaling. Moreover, activation of PI3K/Akt-signaling has been known to exhibit neuroprotective effects in vivo and in vitro PD models [1,11,18].

Because the effects of discrete treatment with MLX on TH levels and Akt phosphorylation were not investigated in this study, whether MLX itself activates TH levels and Akt phosphorylation in mice has not been clarified. However, our previous data [22,23] have indicated that MLX would be most unlikely to affect the Akt signaling by itself, because there was no difference in phosphorylation between MLX-treated and untreated cells. In addition, rats treated with MLX have not shown hyperactive behavior [27]. When Akt phosphorylation in dopaminergic neurons is activated, the animal should

show hyperactive behavior. Moreover, the administration of MLX has not caused dyskinesia-like behavior in osteoarthritis patients [5]. Taken together, the preceding results indicate that MLX itself should not increase in TH levels and Akt phosphorylation. Further studies are needed to clarify the effects of MLX on its own.

All in all, the present results cannot exclude inhibition by COX inhibitors (including oxicams) as disease-modifying drugs for PD treatment, since both apoptotic and inflammatory pathways are involved in PD-related dopaminergic neurodegeneration [3]. As far as we can interpret our present findings, oxicam NSAIDs may achieve potential clinical benefits in the treatment of parkinsonian patients. Furthermore, these drugs could immediately be used for PD treatment if their clinical outcomes are confirmed in clinical trials.

Acknowledgements

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Figure legends

Fig. 1. Neuroprotective effect of meloxicam (MLX) on bradykinesia in the chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Parkinson's disease (MPTP-PD) mouse model. Time-intervals of Tturn (A) and TLA (B) in the pole test were used as indices for comparing bradykinesia in the control (n=8), MPTP-treated (n=12) and MPTP/MLX-treated (n=14) groups (see details described in the 'Materials and methods'). Data are expressed as the mean ± S.E.M. Differences where $P < 0.001$ (*) were considered statistically significant by the Bonferroni post hoc test for one-factorial ANOVA.

Fig. 2. Ameliorative effect of meloxicam (MLX) on dopaminergic neurodegeneration in the striatum (A) and midbrain (B) of the chronic MPTP-PD mouse model. Dopaminergic degenerations in the control (n=8), MPTP-treated (n=12) and MPTP/MLX-treated (n=14) groups were manifested if reduced TH protein levels were detected by the Western blot. Representative images of TH (A) and the reference β-actin (B) are shown accordingly. Data are expressed as the mean ± S.E.M. Differences where $P < 0.05$ (*), < 0.01 (**) and < 0.001 (***) were considered statistically significant by the post hoc Bonferroni test for one-factorial ANOVA. Cases with N.S. are those where statistical significance was not established by either test. See Fig. 1 for abbreviations used.

Fig. 3. Effects of MPTP and meloxicam (MLX) on phosphorylated Akt at Ser 473 or pAkt473 (A) and total Akt (B) levels, and the ratio of pAkt473/total Akt (C) in the midbrain of the chronic MPTP-PD mouse model. Representative images of phosphorylated

Akt (A) and total Akt (B) are indicated accordingly. Data of the control (n=8), MPTP-treated (n=12) and MPTP/MLX-treated (n=14) groups are expressed as the mean ± S.E.M. Differences where $P < 0.05$ (*) and < 0.001 (**) were considered significant by the post hoc Bonferroni test for one-factorial ANOVA. Cases with N.S. are those where statistical significance was not established by the either test. See Fig. 1 for abbreviations used.

Fig.1

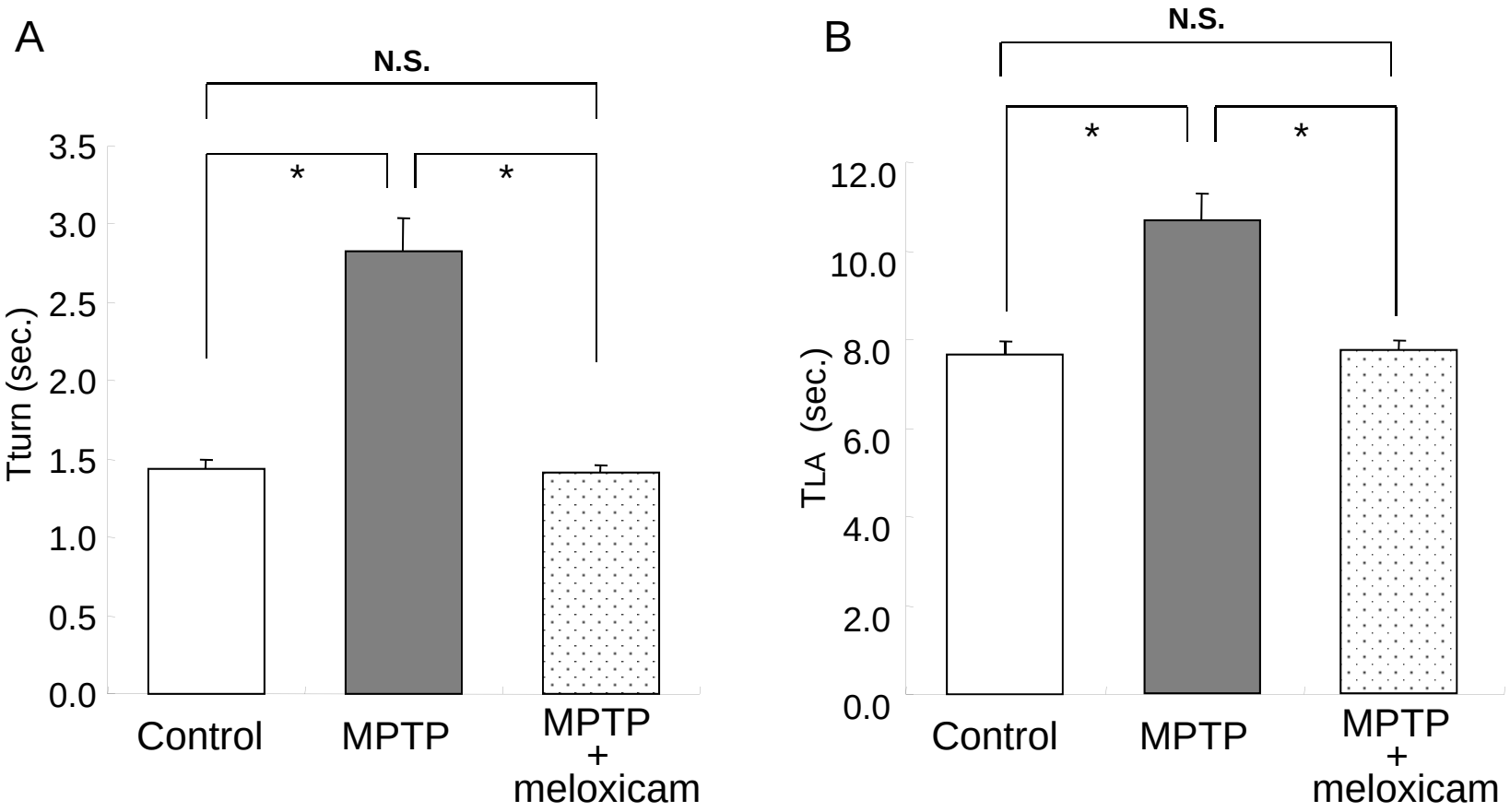
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Fig.2

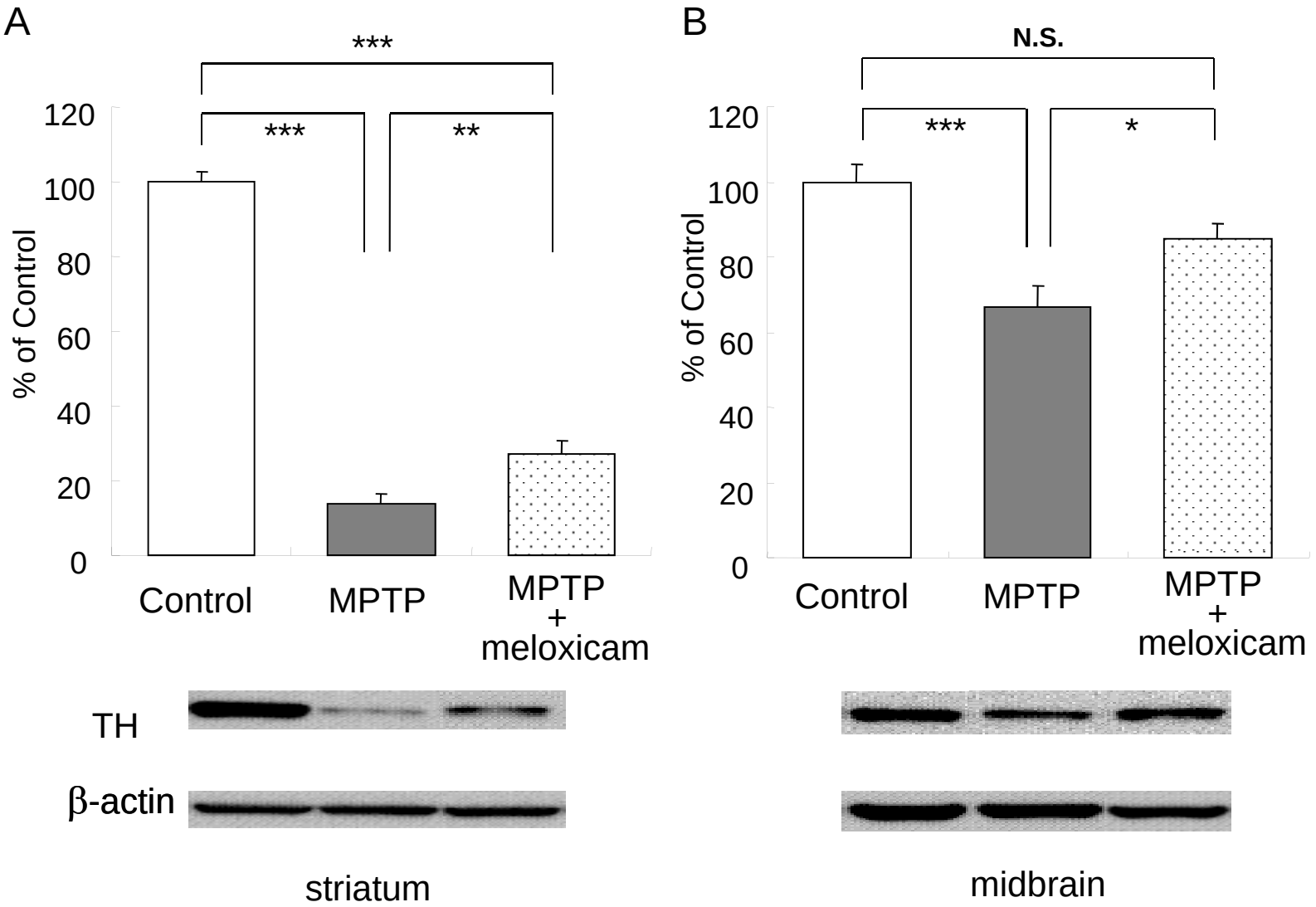
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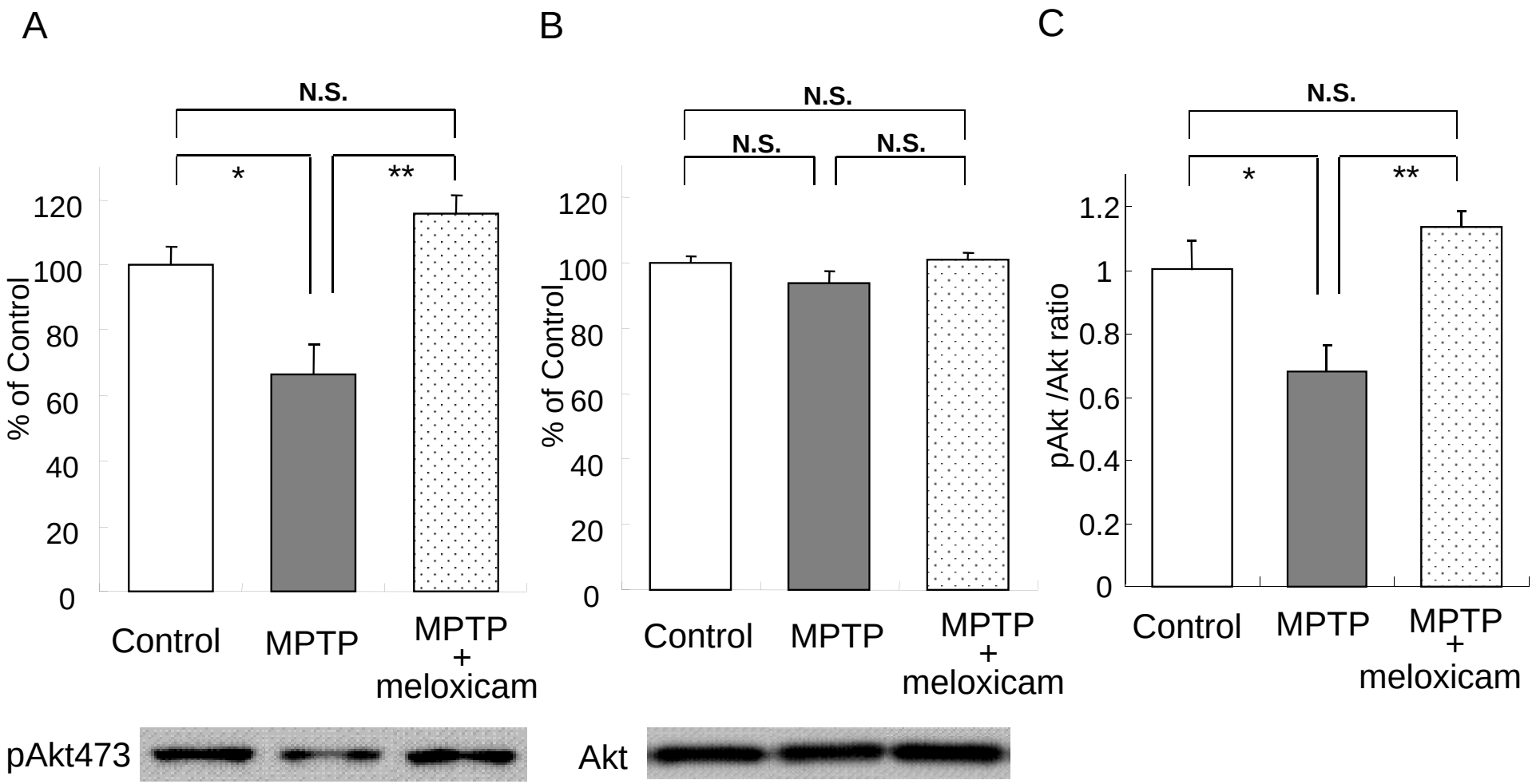
Fig.3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