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TRPV1 on astrocytes rescues nigral dopamine neurons in Parkinson's disease via CNTF

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Currently there is no neuroprotective or neurorestorative therapy for Parkinson's disease. Here we report that transient receptor potential vanilloid 1 (TRPV1) on astrocytes mediates endogenous production of ciliary neurotrophic factor (CNTF), which prevents the active degeneration of dopamine neurons and leads to behavioural recovery through CNTF receptor alpha (CNTFRα) on nigral dopamine neurons in both the MPP^+-lesioned or adeno-associated virus α-synuclein rat models of Parkinson's disease. Western blot and immunohistochemical analysis of human post-mortem substantia nigra from Parkinson's disease suggests that this endogenous neuroprotective system (TRPV1 and CNTF on astrocytes, and CNTFRα on dopamine neurons) might have relevance to human Parkinson's disease. Our results suggest that activation of astrocytic TRPV1 activates endogenous neuroprotective machinery *in vivo* and that it is a novel therapeutic target for the treatment of Parkinson's disease.

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Introduction

Parkinson's disease is a common neurodegenerative disorder characterized by the loss of dopamine neurons in the substantia nigra pars compacta (SNpc) and the appearance of fibrillary aggregates of insoluble α-synuclein (encoded by *SNCA*) called Lewy bodies (Dauer and Przedborski, 2003; Braak *et al.*, 2004). Various Parkinson's disease animal models generated by administration of toxins including 1-methyl-4-phenylpyridinium (MPP$^+$) (Park *et al.*, 2012), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Chung *et al.*, 2010), or 6-hydroxydopamine (6-OHDA) (Drinkut *et al.*, 2012) or α-synuclein over-expression through viral transduction in rats and genetic methods in mice (Kirik *et al.*, 2002; Chesselet, 2008; Decressac *et al.*, 2012) have been used to identify therapeutic targets and test disease-modifying therapies. Although little is known about the cause of Parkinson's disease, major concerns include the loss of dopamine neurons and the subsequent depletion of striatal dopamine, which causes motor abnormalities such as resting tremor, bradykinesia and rigidity (Dauer and Przedborski, 2003; Savitt *et al.*, 2006). A role for non-neuronal cells such as astrocytes in producing neuropathological or neuroprotective functions in Parkinson's disease is becoming increasingly recognized (Block *et al.*, 2007; Rappold and Tieu, 2010).

Astrocytes are the most abundant glial cells in the mammalian brain and they can play both beneficial and detrimental roles in Parkinson's disease (Vila *et al.*, 2001; Rappold and Tieu, 2010; Episcopo *et al.*, 2013). Astrocytes confer neuroprotection by producing neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) (Nakagawa and Schwartz, 2004; Aron and Klein, 2011), mesencephalic astrocyte-derived neurotrophic factor (MANF) (Shen *et al.*, 2012) and ciliary neurotrophic factor (CNTF) (Fischer *et al.*, 2008).

Transient receptor potential vanilloid 1 (TRPV1), the capsaicin receptor, is involved in pain perception and is highly expressed in sensory neurons (Gunthorpe and Szallasi, 2008). TRPV1 is also present in the brain where it may play a role in modulating neuronal function (Starowicz *et al.*, 2008; Kauer and Gibson, 2009), controlling motor behaviour (Lee *et al.*, 2006; Morgese *et al.*, 2007; Gonzalez-Aparicio and Moratalla, 2014), and regulating neuroinflammation (Park *et al.*, 2012). The activation of TRPV1 can be achieved by systemic administration of blood–brain barrier-permeable capsaicin (Guler *et al.*, 2012). Here we show that capsaicin activation of TRPV1 on astrocytes produces endogenous CNTF *in vivo*, which prevents degeneration of dopamine neurons by acting through CNTF receptor alpha (CNTFRα) on dopamine neurons in animal models of Parkinson's disease. This endogenous neuroprotective system (TRPV1 and CNTF on astrocytes, and CNTFRα on dopamine neurons) could be harnessed as a novel beneficial therapeutic target for the treatment of Parkinson's disease.

Materials and methods

Animals

All experiments were done in accordance with the approved animal protocols and guidelines established by Kyung Hee University. Female Sprague–Dawley rats (10 weeks of age, 240–270 g) were housed under a 12:12 h light:dark cycle at an ambient temperature of 22°C. Water and rat chow were available *ad libitum*.

Stereotaxic surgery

Stereotaxic surgery under chloral hydrate was performed as described (Paxinos, 1998; Park et al., 2012). Using coordinates relative to the bregma, stereotaxic injections of MPP^+ (right medial forebrain bundle; A/P −3.6, M/L −2.0, D/V −7.5; MPP^+, 7.4 µg in 2 µl phosphate-buffered saline, Sigma) (Park et al., 2012), or α-synuclein [kindly gifted from Michael J. Fox Foundation]; right substantia nigra (SN); A/P −5.3, M/L −2.3, D/V −7.6; AAV2-eGFP, AAV2-α-synuclein; 0.2 µl/ min, total 2 µl; adeno-associated virus (AAV) virus used here is driven by the chicken β-actin promoter (Kirik et al., 2002), lentivirus (right SN; shTRPV1, shCtrl; 0.2 µl/ min, total 3 µl), CNTFRα neutralizing antibody (right SN; R&D, AF-303-NA; 0.01 µg/µl, 0.2 µl/ min, total 2 µl) and respective control were done according to the atlas of Paxinos and Watson (Paxinos, 1998).

Capsaicin injection

Capsaicin (1 mg/kg, intraperitoneally; a single injection/day for 7 days, Sigma) (Veldhuis et al., 2003; Park et al., 2012) was injected at 1 week and 1 day post MPP^+, and at 7 weeks and 1 day post α-synuclein. Lentivirus was injected immediately after MPP^+ injection.

Rotational behaviour test

D-Amphetamine (5 mg/kg, intraperitoneally) was used to monitor ipsilateral rotations in rats with unilaterally lesioned nigrostriatal dopamine neurons (Jin and Iacovitti, 1995). The ipsilateral rotations were counted for 1 h at 1, 2 or 6 weeks post MPP^+, and 7 or 8 weeks post α-synuclein.

Stereological estimation

As previously described (West et al., 1991; Choi et al., 2003; Bartus et al., 2011; Park et al., 2012), the total number of TH^+ neurons was counted in the various animal groups using the optical fractionator method performed on a brightfield microscope (Olympus Optical, BX51) using Stereo Investigator software (MBF Bioscience). This unbiased stereological method of cell counting is not affected by either the reference volume (SNpc) or the size of the counted elements (neurons).

Morphological analysis

Optical densities of the TH^+ striatal fibres were measured using Science Lab 2001 Image Gauge (Fujifilm) (Nam et al., 2015).

ImageJ analysis

Imaging data were analysed in ImageJ (National Institutes of Health) as described recently (Lee et al., 2010). ImageJ with co-localization plugin was used to quantify immunofluorescence and with colour deconvolution plugin was used to quantify chromogenic signal intensity on image.

Western blot

The SN area was rapidly removed and western blot analysis was performed as previously described (Choi et al., 2003; Shi et al., 2012). The following primary antibodies and dilutions were used: rabbit TRPV1 (1:1000, Alomone labs), mouse anti-CNTF (1:1000, Millipore), mouse anti-GFAP (1:500, Sigma), mouse anti-tyrosine hydroxylase (TH, 1:1000, Millipore), rabbit anti-phosphorylation Ser31 tyrosine hydroxylase (1:1000, Millipore), and mouse anti-beta-actin (1:5000, Abcam). The following secondary antibodies and dilutions were used; horseradish peroxidase-conjugated anti-rabbit or mouse IgG (1:5000, Bethyl).

Western blot analysis of human brain tissues

Human brain tissues (Supplementary Tables 1 and 2) from SN and cortex were homogenized in lysis buffer [10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10 mM Na-β-glycerophosphate, 1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, Phosphatase Inhibitor Mixture I and II (Sigma), and Complete Protease Inhibitor Mixture (Roche)], using a Diax 900 homogenizer (Sigma). After homogenization, samples were rotated at 4°C for 30 min for complete lysis, the homogenate was centrifuged at 52 000 rpm for 20 min, and the resulting fractions were collected. Protein levels were quantified using the BCA Kit (Pierce) with bovine serum albumin (BSA) standards, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with indicated antibodies. Immunoblot signals were visualized with chemiluminescence.

Immunostaining

Human brain tissues [age matched control and Parkinson's disease subjects obtained from the Victoria Brain Bank Network (VBBN), Supplementary Table 3] were deparaffinized in xylene and subjected to citrate antigen retrieval prior to immunohistochemistry (Martins et al., 1999). Tissues sections were washed in cold phosphate-buffered saline (PBS) for 15 min and block with universal blocking solution (0.3% Triton™ X-100, 1% BSA, 0.05% Tween 20, 0.1% cold fish gelatin and 0.05% sodium azide in PBS) for 1 h at room temperature. Primary antibodies of mouse anti-GFAP (1:500, Sigma), rabbit anti-TRPV1 (1:1000, Alomone labs), rabbit anti-CNTF (1:200, Santa-Cruz) and goat anti-CNTFRα (1:200, Santa-Cruz) were diluted in 1–5% BSA or normal goat serum and incubated according to manufacturer recommendations. For fluorescent microscopy, FITC-conjugated-anti-mouse or goat (1:400, Millipore), Cy3-conjugated-anti-rabbit (1:400, Millipore) and Texas Red-conjugated-anti-rabbit (1:400, Vector Laboratories) secondary antibodies with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Vector Laboratories). After washing with PBS, coverslips were mounted on glass slides using mounting media (Vector Laboratories), and analysed using a confocal microscope (LSM 700, Carl Zeiss). For light microscopy, brain tissues were labelled using alkaline phosphatase-conjugated secondary antibodies (Vector Laboratories). After incubation, immunostaining visualized with the peroxidase with Alkaline Phosphatase Substrate Kit III (blue) (Vector Laboratories). To examine the expression of the CNTFRα, brain tissues were used appropriate biotin secondary antibody, followed by avidin-biotin complex (Vector Laboratories) and visualized with 3,3'-diaminobenzidine (DAB) peroxidase substrate solution-Blue (0.05% DAB, 0.05% cobalt chloride, 0.05% nickel ammonium sulphate and 0.015% H_2O_2 in PBS, pH 7.2).

For rat brain, as described (Park *et al.*, 2012), animals were transcardially perfused, fixed and brain tissues (40-μm thick) were processed for immunohistochemical staining. In brief, sections were rinsed in PBS then incubated with the following primary antibodies: mouse anti-α-synuclein (1:1000, Abcam), rabbit anti-TRPV1 (1:1000, Alomone labs), rabbit anti-CNTF (1:200, Santa-Cruz), mouse and rabbit anti-GFAP (1:500, mouse, Sigma; 1:5000, rabbit, Neuromics) for astrocytes, rabbit or mouse anti-tyrosine hydroxylase (TH, 1:2000, rabbit, Pel-Freez; 1:2000; mouse, Millipore) for dopamine neurons and mouse anti-OX42 (1:400, Serotec) and anti-Iba-1 (1:1000, Wako) for microglia. The next day, tissues were rinsed and incubated with FITC-conjugated-anti-rabbit, mouse or goat IgG (1:400, Millipore), Cy3-conjugated-anti-rabbit IgG (1:400, Millipore), Texas Red-conjugated-anti-mouse IgG (1:400, Vector Laboratories), CF405M-conjugated- anti- rabbit or mouse IgG (1:400, Biotium) and 405-conjugated-anti-goat IgG (1:200, Jackson Immunoresearch) 1 h. Stained tissues were viewed using a confocal microscopy (LSM700, Carl Zeiss) or were analysed under a bright-field micro-scope (Olympus).

TRPV1 shRNA and lentivirus production

For plasmid-based short hairpin (sh)RNA expression, the following complementary oligonucleotides were annealed and inserted into the HindIII/BglII sites of pSUPER-GFP vector: gcgcatcttctacttcaac (sense) TTAGCACTG (loop) gttgaagtagaagatgcgc (antisense), corresponding to nucleotide sequence of TRPV1 (Christoph *et al.*, 2008). For lentivirus-based shRNA expression, a lentiviral vector containing TRPV1 gene was constructed by inserting synthetic double-strand oligonucleotides 5'-CGCTGCAGTTGCCAACTTGTCAATGAATTCAAGAGAT TCATTG ACAAGTTGGCAATTTTTGATATCTAGACA-3' into the HpaI–XhoI restriction enzyme sites of the pSicoR-mcherry lentiviral vector. A shLenti construct containing scrambled oligonucleotides: 5'-CGCATAGCGTATGCCGTTTTCAAGAGAA ACGGCATACGCTATGCGATTTTTTC-3' was used as a control.

Statistical analysis

All values are expressed as mean ± standard error of the mean. Statistical significance ($P < 0.05$ for all analysis) was assessed by ANOVA using the Instat 3.05 software package (GraphPad Software, San Diego, CA, USA), followed by Student–Newman–Keuls analyses.

Results

Astrocytic TRPV1-derived CNTF protects degeneration of dopamine neurons from MPP[+] neurotoxicity *in vivo*

To explore the potential function of TRPV1 in Parkinson's disease, the rat unilateral MPP[+]-lesion model of Parkinson's disease was used (Park *et al.*, 2012). The loss of nigral dopamine neurons in the MPP[+] model used here is 40% at 1 week post MPP[+] and 63% at 2 week post MPP[+] (Supplementary Fig. 1).
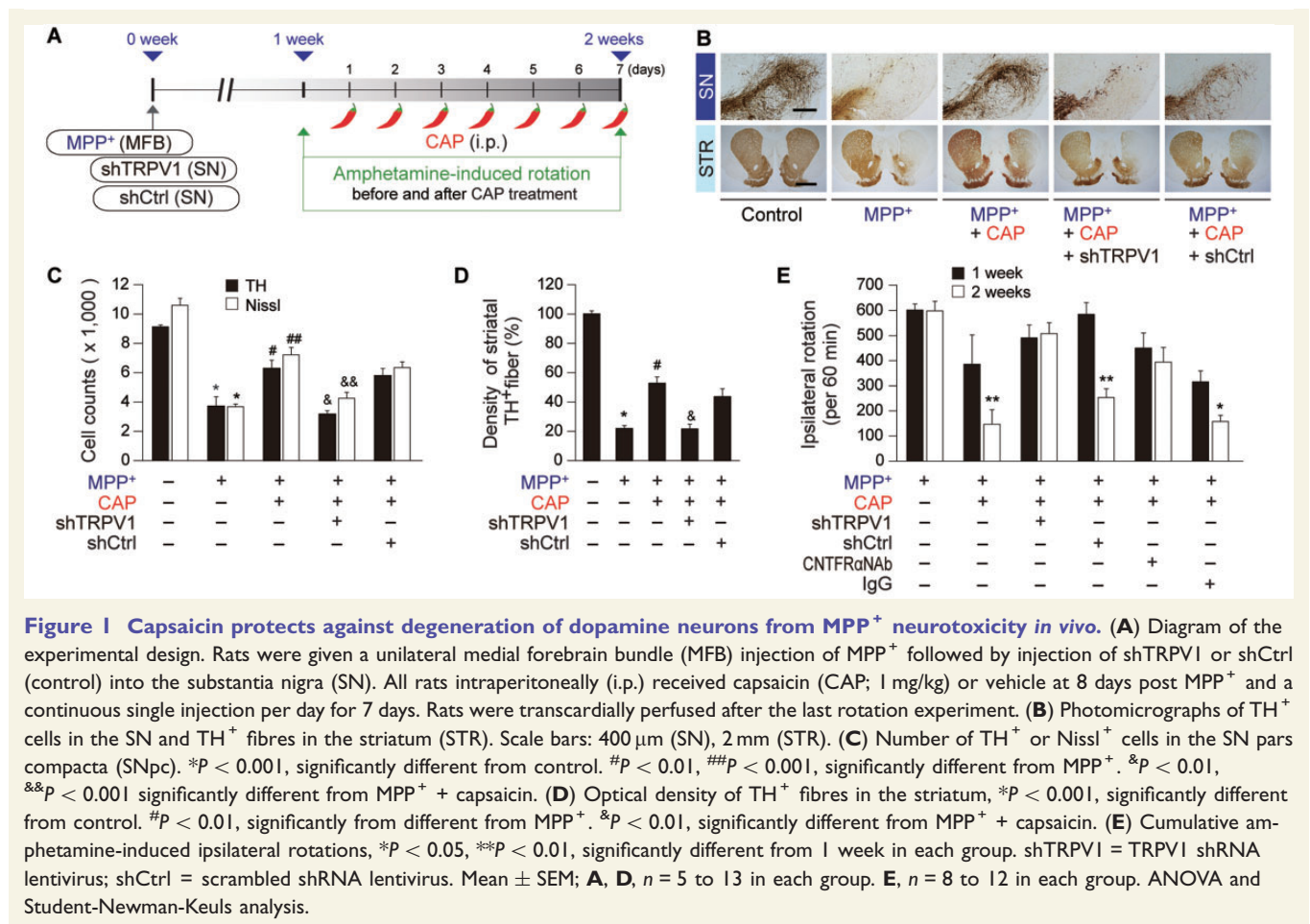
Amphetamine (5 mg/kg, intraperitoneally)-induced ipsilateral rotation (Jin and Iacovitti, 1995) was analysed at 1 week after MPP[+] administration. Rats that exhibited ipsilateral rotations, indicative of an effective lesion were randomly selected for treatment with the TRPV1 agonist, capsaicin (1 mg/kg, intraperitoneally) (Veldhuis *et al.*, 2003; Park *et al.*, 2012) or vehicle each day for 7 days (Fig. 1A). Similar to our recent report (Park *et al.*, 2012), capsaicin prevents the ongoing degeneration in this model.

The number of TH[+] and Nissl^{+} cells as assessed by stereology in the SNpc and the density of TH[+] fibres in the striatum are significantly higher in the MPP[+]-lesioned rats treated with capsaicin, compared with the vehicle-treated rats (Fig. 1B–D). Capsaicin also significantly attenuates amphetamine-induced rotations indicative of behavioural rescue (Fig. 1E). These behavioural and neuroprotective effects were prolonged up to 4 weeks after the last capsaicin treatment (Fig. 2).

To confirm that capsaicin is mediating protection via activation of TRPV1, we selectively inhibited TRPV1 function with a lentivirus carrying a small hairpin-forming interference RNA (shRNA) targeted against TRPV1 (shTRPV1) (Christoph *et al.*, 2008; Lee *et al.*, 2010). This virus also contained DNA encoding a fluorescent marker, mCherry, which permitted visualization of the location and amount of viral infection. When shTRPV1 or control scrambled shRNA (shCtrl) was injected into the rat SN immediately after the unilateral medial forebrain bundle injection of MPP[+] (Fig. 1A), shTRPV1 efficiently reduced TRPV1 protein expression (Supplementary Fig. 2A and B) mainly within astrocytes in the SNpc of MPP[+]-lesioned rats at 1 week post MPP[+] (Supplementary Fig. 2C and F). Compared to GFAP, there was relatively minimal mCherry expression in TH^{+} neurons or OX-42^{+} microglia (Supplementary Fig. 2D–F). Knockdown of TRPV1 exacerbates the MPP[+]-induced loss of TH^{+} neurons as assessed by stereological counting of TH[+] and Nissl^{+} cells (Supplementary Fig. 2G and H), suggesting that TRPV1 might function as endogenous neuroprotective machinery *in vivo*. In the capsaicin-treated MPP[+]-lesioned rats, knockdown of TRPV1 expression by shTRPV1 significantly inhibited capsaicin neuroprotection by reducing the number of TH[+] and Nissl^{+} cells in the SNpc (Fig. 1B and C) and density of TH[+] fibres in the striatum (Fig. 1B–D) compared to the shCtrl-injected rats at 2 weeks post MPP[+]. Accompanying the lack of neuroprotection is an attenuation of the effects of capsaicin on amphetamine-induced ipsilateral rotations compared to the shCtrl injection (Fig. 1E). Thus, capsaicin, via activation of TRPV1 on astrocytes, produces a functional recovery and protects dopamine neurons *in vivo* from MPP[+] toxicity.

Expression of TRPV1 was analysed in GFAP[+] astrocytes, TH^{+} neurons and OX-42^{+} microglia at 1 week post MPP[+]. Expression of GFAP and TRPV1 in GFAP[+] astrocytes was significantly higher in the rat SNpc at 1 week

Figure 1 Capsaicin protects against degeneration of dopamine neurons from MPP⁺ neurotoxicity *in vivo*. (**A**) Diagram of the experimental design. Rats were given a unilateral medial forebrain bundle (MFB) injection of MPP⁺ followed by injection of shTRPV1 or shCtrl (control) into the substantia nigra (SN). All rats intraperitoneally (i.p.) received capsaicin (CAP; 1 mg/kg) or vehicle at 8 days post MPP⁺ and a continuous single injection per day for 7 days. Rats were transcardially perfused after the last rotation experiment. (**B**) Photomicrographs of TH⁺ cells in the SN and TH⁺ fibres in the striatum (STR). Scale bars: 400 μm (SN), 2 mm (STR). (**C**) Number of TH⁺ or Nissl⁺ cells in the SN pars compacta (SNpc). *$P < 0.001$, significantly different from control. #$P < 0.01$, ##$P < 0.001$, significantly different from MPP⁺. &$P < 0.01$, &&$P < 0.001$ significantly different from MPP⁺ + capsaicin. (**D**) Optical density of TH⁺ fibres in the striatum, *$P < 0.001$, significantly different from control. #$P < 0.01$, significantly from different from MPP⁺. &$P < 0.01$, significantly different from MPP⁺ + capsaicin. (**E**) Cumulative amphetamine-induced ipsilateral rotations, *$P < 0.05$, **$P < 0.01$, significantly different from 1 week in each group. shTRPV1 = TRPV1 shRNA lentivirus; shCtrl = scrambled shRNA lentivirus. Mean ± SEM; **A**, **D**, $n = 5$ to 13 in each group. **E**, $n = 8$ to 12 in each group. ANOVA and Student-Newman-Keuls analysis.

post MPP⁺ compared to the control, whereas TRPV1 in TH⁺ neurons was significantly reduced and TRPV1 in OX-42⁺ microglia was relatively unchanged (Fig. 3A–D). Western blot analysis shows a higher expression of GFAP with total TRPV1 levels unchanged in the SN, whereas TH levels are significantly reduced by 48% at 1 week post MPP⁺ compared to the control (Fig. 3E and F). Taken together these results indicate that following MPP⁺-induced degeneration of dopamine neurons there is a reduction of TRPV1 in TH⁺ neurons and a significant upregulation in GFAP⁺ astrocytes, which is consistent with capsaicin acting primarily on astrocytic TRPV1 receptors (Fig. 1).

As capsaicin seems to be primarily exerting its protective function via astrocytic TRPV1 receptors, we wondered if it could exert neuroprotection via CNTF, which is expressed in astrocytes upon brain injury (Stockli *et al.*, 1989; Lee *et al.*, 1998; Park *et al.*, 2000; Leibinger *et al.*, 2009; Kang *et al.*, 2012). Immunohistochemical analysis reveals a significant increase in expression of CNTF and particular, in GFAP⁺ astrocytes in the SNpc of capsaicin-treated MPP⁺-lesioned rat brain (Fig. 4A–C) compared to vehicle treated control at 2 weeks post MPP⁺ (Fig. 4A–C). shTRPV1 attenuated capsaicin-induced expression of CNTF and CNTF in GFAP⁺ astrocytes (Fig. 4A–C) in the SNpc of

MPP⁺-lesioned rats compared to shCtrl (Fig. 4A–C). Immunohistochemical analysis indicates that the CNTFRα is expressed in TH⁺ cells in the SNpc of intact or MPP⁺-lesioned rats (Fig. 4D–F). Accordingly, we tested the effect of CNTFRα on the survival of dopamine neurons. CNTFRα neutralizing antibody was unilaterally injected to block CNTF actions in the ipsilateral rat SNpc at 1 week after the unilateral medial forebrain bundle injection of MPP⁺ (Fig. 5A). The capsaicin-induced increase in number of TH⁺ and Nissl⁺ cells in the SNpc and density of TH⁺ fibres in the striatum was significantly reduced in MPP⁺-lesioned rats treated with CNTFRα neutralizing antibody compared with the non-specific IgG-treated rats as a control (Fig. 5B and C). Accompanying the lack of neuroprotection is an attenuation of the effects of capsaicin on amphetamine-induced ipsilateral rotations compared with the IgG injection (Fig. 1E).

CNTF produced by astrocytes can stimulate TH enzyme activity via phosphorylation of TH at Ser31 (Shi *et al.*, 2012) *in vivo*, eventually contributing to functional recovery. To test this possibility, western blot analysis was performed to measure changes in CNTF levels and examine the state of TH phosphorylation at Ser31 (pTHser31) (Shi *et al.*, 2012). In capsaicin-treated MPP⁺-lesioned
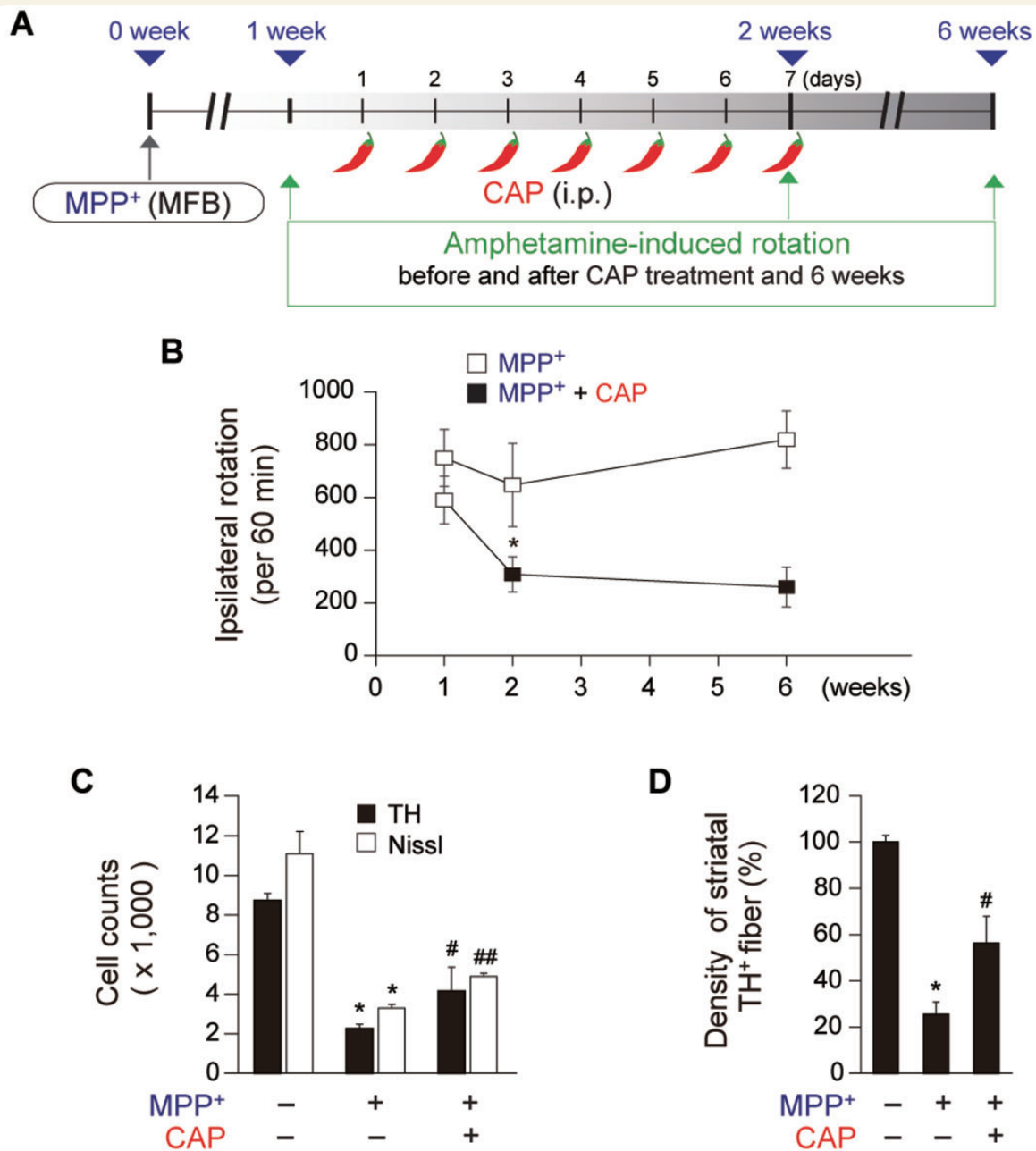
Figure 2 Prolonged neuroprotective effects of capsaicin. (A) Diagram of the experimental design. Rats were given a unilateral medial forebrain bundle (MFB) injection of MPP$^+$. All rats intraperitoneally (i.p.) received capsaicin (CAP; 1 mg/kg) or vehicle at 8 days post MPP$^+$ and a continuous single injection per day for 7 days. Amphetamine-induced rotation was analysed at 1, 2, or 6 weeks post MPP$^+$ and rats were transcardially perfused after the last rotation experiment. **(B)** Cumulative amphetamine-induced ipsilateral rotations, *$P < 0.05$, significantly different from 2 weeks MPP$^+$. **(C)** Number of TH$^+$ or Nissl$^+$ stained cells in the SNpc, *$P < 0.001$, significantly different from control, #$P < 0.05$, ##$P < 0.01$, significantly different from MPP$^+$. **(D)** Optical density of TH$^+$ fibres in the striatum (STR), *$P < 0.01$, significantly from different control, #$P < 0.05$, significantly different from MPP$^+$. Mean ± SEM; **B–D**, $n = 3$–7 in each group. ANOVA and Student-Newman-Keuls analysis.

rats, CNTF levels are increased (Fig. 5D and E) compared to vehicle-treated MPP$^+$-lesioned rats consistent with the immunohistochemical data (Fig. 4A–C). In parallel, in the capsaicin-treated MPP$^+$-lesioned rat, pTHser31 levels are increased in the SN (Fig. 5D and E) compared to vehicle-treated MPP$^+$-lesioned rats, indicating activation of TH

enzyme activity. In the capsaicin-treated MPP$^+$-lesioned rat, total TH levels are also increased (Fig. 5D and E) compared to vehicle-treated MPP$^+$-lesioned rats, reflecting increased survival of dopamine neurons in the SN. CNTFRα neutralizing antibody (Fig. 5D and E) prevents the increase in total TH levels and pTHser31 levels
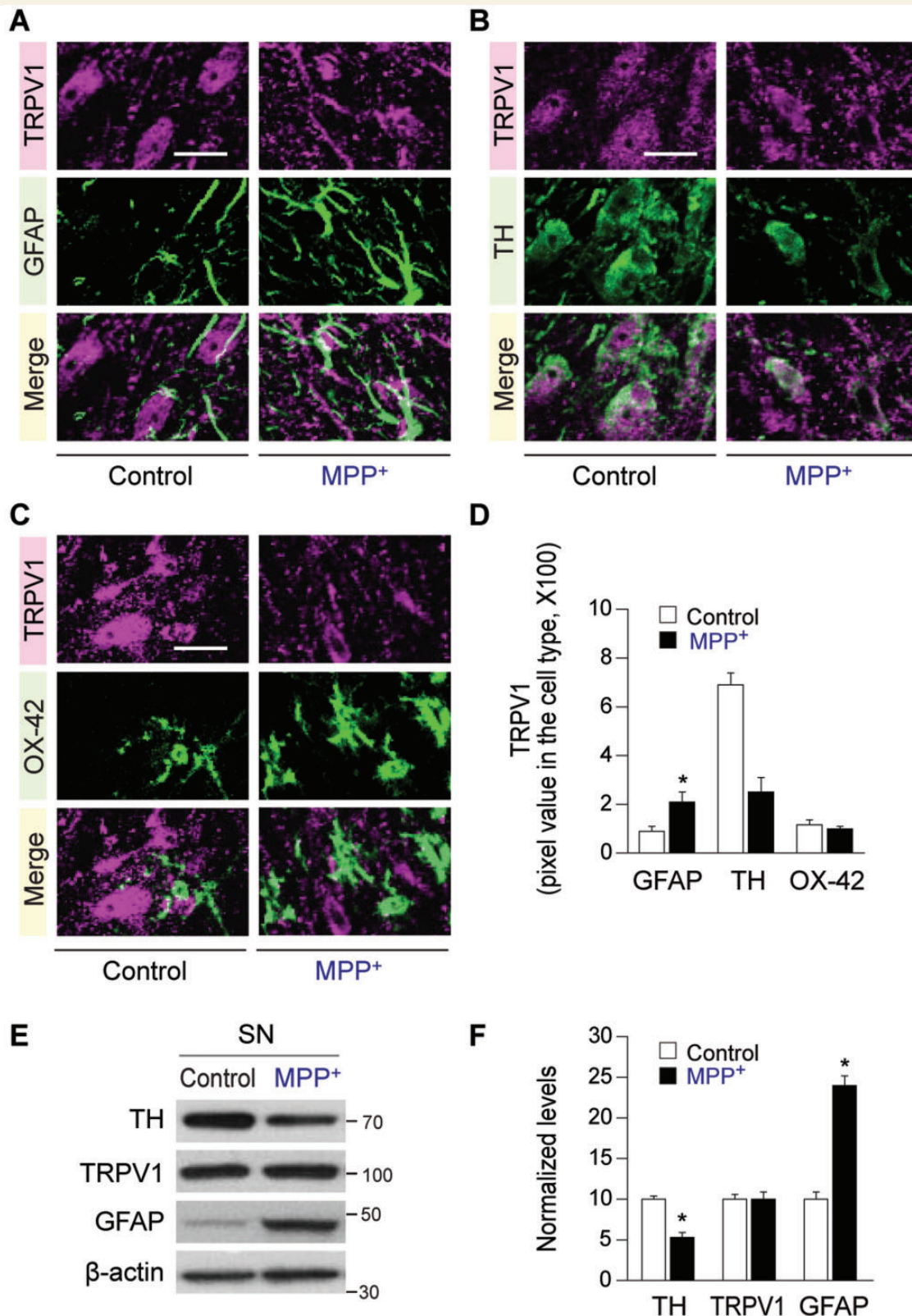
Figure 3 TRPV1 expression in the substantia nigra of MPP⁺-lesioned rat. The rats were given a unilateral medial forebrain bundle (MFB) injection of MPP⁺ and brain tissues were processed for immunohistochemical and western blot analysis at 1 week post MPP⁺. (**A–D**) Fluorescence images of TRPV1 (magenta; **A**) and GFAP (green; **A**), or TRPV1 (magenta; **B**) and TH (green; **B**) or TRPV1 (magenta; **C**) and OX-42 (green; **C**) and both images are merged (yellow; **A–C**) in the SNpc of MPP⁺-lesioned rat brain. (**D**) Quantification of TRPV1 expression in each cell type, *$P < 0.01$, **$P < 0.001$, significantly different from control. (**E** and **F**) Western blot analysis of TH, TRPV1 and GFAP (**E**), and quantification (**F**) in the SN of MPP⁺-lesioned rat brain, *$P < 0.001$, significantly different from control. Scale bars = 20 μm. Mean ± SEM; D, $n = 6$–7; F, $n = 4$–6. ANOVA and Student-Newman-Keuls analysis.
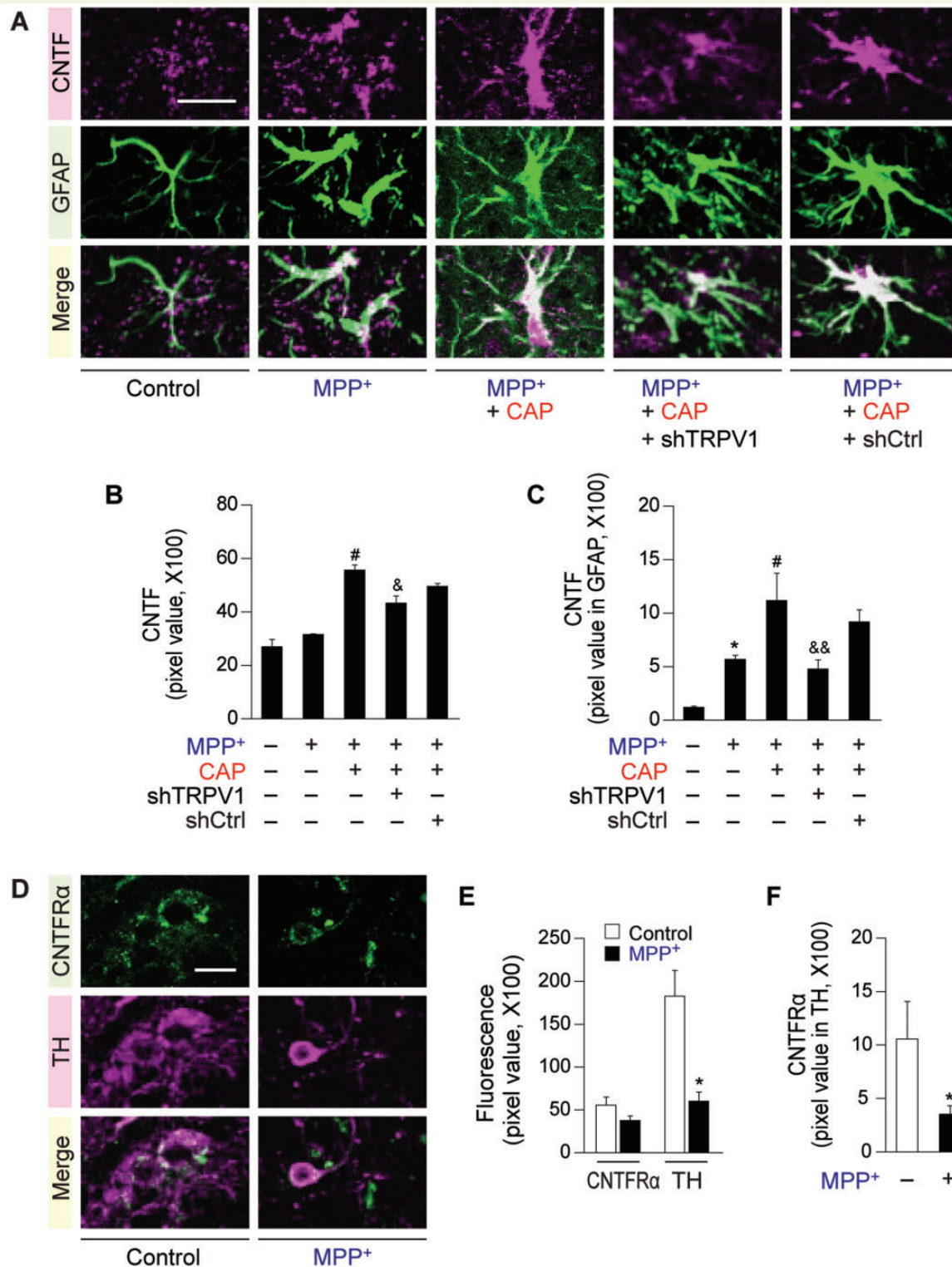
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Figure 4 CNTF and CNTFRα expression in the substantia nigra of MPP⁺-lesioned rat. Brain sections adjacent to those used in Fig. 1 were immunostained. (**A**) Fluorescence images of CNTF (magenta) and GFAP (green) and both images are merged in the SNpc of MPP⁺-lesioned rat brain. Florescence images of CNTF (from green to magenta) and GFAP (from blue to green) were pseudo-coloured in MPP⁺ + capsaicin + shTRPV1- or MPP⁺ + capsaicin + shCtrl-treated tissues. (**B** and **C**) Quantification of CNTF (**B**) or CNTF expression in GFAP⁺ astrocytes (**C**) of rat SNpc at 2 weeks post MPP⁺, *$P < 0.001$, significantly different from control, #$P < 0.01$, significantly different from MPP⁺, &$P < 0.01$, &&$P < 0.001$, significantly different from MPP⁺ + capsaicin. (**D**) Fluorescence images of CNTFRα (green) and TH⁺ dopamine neurons (magenta), and both images are merged in the rat SNpc at 1 week after a unilateral medial forebrain bundle (MFB) injection of MPP⁺. (**E** and **F**) Quantification of CNTFRα or TH expression (**E**) and CNTFRα expression in TH⁺ dopamine neurons (**F**), *$P < 0.05$, significantly different from control. Scale bars = 20 μm. Mean ± SEM; **B** and **C**, $n = 4$–8. **E** and **F**, $n = 5$. ANOVA and Student-Newman-Keuls analysis.
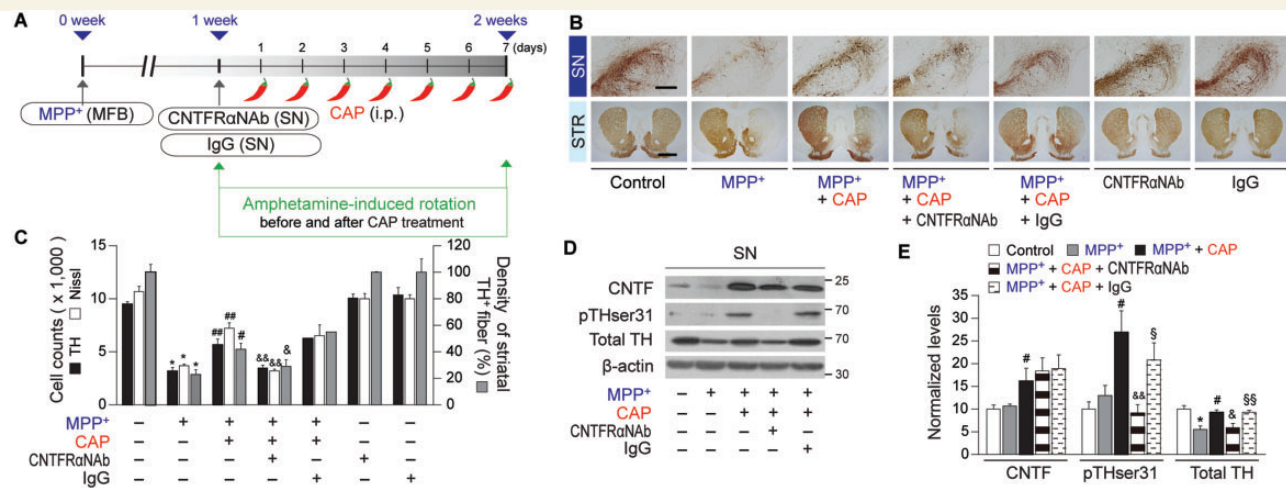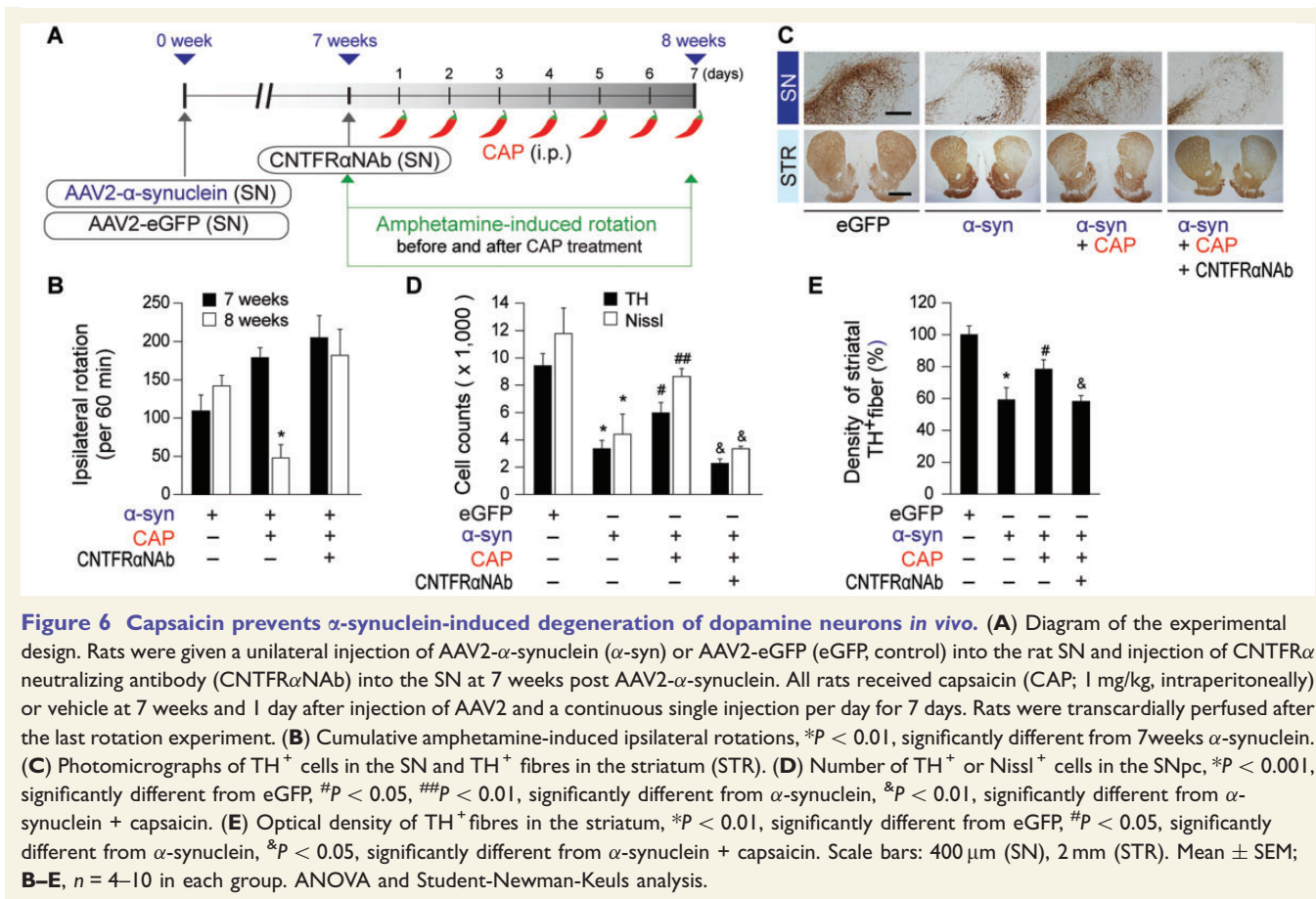
Figure 5 Astrocytic TRPV1 induced CNTF exerts neuroprotection on dopamine neurons *in vivo*. (**A**) Diagram of the experimental design showing various treatments as indicated. After the last rotation experiment, brain tissues were prepared for immunohistochemical staining or western blot analysis. (**B**) Photomicrographs of TH$^+$ cells in the SN and TH$^+$ fibres in the striatum (STR). (**C**) Number of TH$^+$ or Nissl$^+$ cells in the SNpc, and optical density of TH$^+$ fibres in the striatum, *$P < 0.001$, significantly different from control, #$P < 0.05$, ##$P < 0.001$, significantly different from MPP$^+$, &$P < 0.05$, &&$P < 0.01$, significantly different from MPP$^+$ + capsaicin. (**D** and **E**) Western blot analysis (**D**), and quantification (**E**) in the rat substantia nigra (SN) at 2 weeks post MPP$^+$, *$P < 0.01$, significantly different from control, #$P < 0.01$, significantly different from MPP$^+$, &$P < 0.01$, &&$P < 0.001$, significantly different from MPP$^+$ + capsaicin, §$P < 0.05$, §§$P < 0.01$, significantly different from MPP$^+$ + capsaicin + CNTFα neutralizing antibody (CNTFαNAb). Scale bars = 400 μm (SN); 2 mm (STR). Mean ± SEM; **C**, $n = 3$–13 in each group. **D** and **E**, $n = 5$–7 in each group. ANOVA and Student-Newman-Keuls analysis.

compared control IgG-treated rats (Fig. 5D and E). In parallel, CNTFRα neutralizing antibody attenuated the effects of capsaicin on amphetamine-induced ipsilateral rotations compared with the IgG injection (Fig. 1E). Taken together these results provide evidence that astrocyte-derived endogenous CNTF can act on CNTFRα and prevent MPP$^+$-induced degeneration of dopamine neurons and stimulate TH enzyme activity *in vivo*.

Astrocytic TRPV1-derived CNTF prevents degeneration of dopamine neurons in an α-synuclein model of Parkinson's disease

To determine whether these findings extend to a genetic model of Parkinson's disease, the rat unilateral α-synuclein lesion model of Parkinson's disease was used (Kirik *et al.*, 2002). Recombinant adeno-associated virus serotype 2 (AAV2) containing the human wild-type *SNCA* gene (AAV2-α-synuclein or α-synuclein) or enhanced green fluorescent protein (AAV2-eGFP or eGFP) were stereotaxically injected into the SN. At 7 weeks post α-synuclein, immunohistochemical analysis shows an efficient transduction of α-synuclein into the nigrostriatal dopamine neurons (Supplementary Fig. 3A and B). The loss of nigral TH$^+$ neurons in the α-synuclein model used here is 44% at 7 weeks and 64% at 8 weeks post α-synuclein (Supplementary Fig. 3C).

In α-synuclein-injected rats, expression pattern of TRPV1 and CNTF on astrocytes or of CNTFRα on dopamine neurons was the same as those of MPP$^+$-treated rats compared to eGFP (Supplementary Fig. 3D–L). At 7 weeks post AAV2-α-synuclein rats were treated with capsaicin (intraperitoneal 1 mg/kg) or vehicle (Fig. 6A). In α-synuclein-lesioned rats, capsaicin treatment significantly enhanced the expression of CNTF (Supplementary Fig. 3J and K) and CNTF in GFAP$^+$ astrocytes (Supplementary Fig. 3J and L) in the SNpc compared to vehicle-treated control at 8 weeks post α-synuclein, similar to capsaicin treatment in the MPP$^+$-lesioned model (Fig. 4). Capsaicin-treatment of α-synuclein-lesioned rats significantly reduces amphetamine-induced rotations at 8 weeks post α-synuclein compared to vehicle-treated α-synuclein-lesioned rats (Fig. 6B). Accompanying with behavioural improvement, capsaicin increases number of TH$^+$ and Nissl$^+$ cells in the SNpc and density of TH$^+$ fibres in the striatum at 8 weeks post α-synuclein compared with vehicle-treated rats (Fig. 6C–E). EGFP (control) had no effects on amphetamine-induced rotations (data not shown) and degeneration of dopamine neurons (Fig. 6C–E). CNTFRα neutralizing antibody (Fig. 6A) prevents the behavioural recovery (Fig. 6B) and the rescue of TH$^+$ and Nissl$^+$ cells in the SNpc and density of TH$^+$ fibres in the striatum (Fig. 6C-E). Thus, consistent with the toxin (MPP$^+$)-based Parkinson's disease model, TRPV1 activated by capsaicin contributes to production of CNTF from astrocytes and prevents α-synuclein-induced degeneration of dopamine neurons *in vivo*.

Figure 6 Capsaicin prevents α-synuclein-induced degeneration of dopamine neurons *in vivo*. (**A**) Diagram of the experimental design. Rats were given a unilateral injection of AAV2-α-synuclein (α-syn) or AAV2-eGFP (eGFP, control) into the rat SN and injection of CNTFRα neutralizing antibody (CNTFRαNAb) into the SN at 7 weeks post AAV2-α-synuclein. All rats received capsaicin (CAP; 1 mg/kg, intraperitoneally) or vehicle at 7 weeks and 1 day after injection of AAV2 and a continuous single injection per day for 7 days. Rats were transcardially perfused after the last rotation experiment. (**B**) Cumulative amphetamine-induced ipsilateral rotations, $*P < 0.01$, significantly different from 7 weeks α-synuclein. (**C**) Photomicrographs of TH^+ cells in the SN and TH^+ fibres in the striatum (STR). (**D**) Number of TH^+ or $Nissl^+$ cells in the SNpc, $*P < 0.001$, significantly different from eGFP, $^\#P < 0.05$, $^{\#\#}P < 0.01$, significantly different from α-synuclein, $^\&P < 0.01$, significantly different from α-synuclein + capsaicin. (**E**) Optical density of TH^+ fibres in the striatum, $*P < 0.01$, significantly different from eGFP, $^\#P < 0.05$, significantly different from α-synuclein, $^\&P < 0.05$, significantly different from α-synuclein + capsaicin. Scale bars: 400 μm (SN), 2 mm (STR). Mean ± SEM; **B–E**, $n = 4$–10 in each group. ANOVA and Student-Newman-Keuls analysis.

Expression of TH, TRPV1, GFAP, CNTF and CNTFRα in the substantia nigra of human Parkinson's disease brain

To ascertain whether these findings might have relevance to Parkinson's disease, human post-mortem SN from control versus Parkinson's disease patients was examined. Western blot analysis reveals a significant increase in TRPV1 and GFAP levels in Parkinson's disease SN versus control SN (Fig. 7A and B). In addition, there is a significant increase in the levels of CNTF and CNTFRα of Parkinson's disease SN versus control SN (Fig. 7C and D). These changes seem to be specific to the SN as there is no significant difference in the levels of TRPV1, CNTF, CNTFRα and GFAP in the cortex, which is relatively unaffected in Parkinson's disease (Fig. 7E and F). Immunohistochemical analysis reveals a higher expression of TRPV1 in $GFAP^+$ astrocytes (Fig. 7G and H) and a higher expression of CNTF in $GFAP^+$ astrocytes in the SNpc of human Parkinson's disease (Fig. 7I and J). Immunohistochemical analysis also reveals a significant increase in levels of CNTFRα expression in each remaining dopamine neuron in the SNpc of Parkinson's disease brain compared to human control brain (Fig. 7K–O). Overall these changes are similar to the changes observed in the MPP^+ and the α-synuclein rat models of Parkinson's disease.

Discussion

The major finding of this manuscript is the observation that TRPV1 activation by capsaicin produces endogenous CNTF, which acts on CNTFRα on nigral dopamine neurons protecting against degeneration of dopamine neurons and eliciting behavioural recovery in the MPP^+ rat model of Parkinson's disease (Supplementary Fig. 4). This occurs through activation of TRPV1 on astrocytes since the protection afforded by capsaicin in the MPP^+ rat model of Parkinson's disease is reduced by knockdown of TRPV1 in astrocytes. In addition, TRPV1 activation by capsaicin also protects against loss of dopamine neurons in the α-synuclein rat model of Parkinson's disease and provides behavioural recovery via increases in astrocyte-derived CNTF levels acting on CNTFRα on nigral dopamine neurons. These findings may have relevance to human Parkinson's disease as TRPV1 and CNTF levels in $GFAP^+$ astrocytes and CNTFRα on $neuromelanin^+$ nigral dopamine neurons are increased in human Parkinson's disease SN similar to the MPP^+ and the α-synuclein rat models of Parkinson's disease. TRPV1 and CNTF
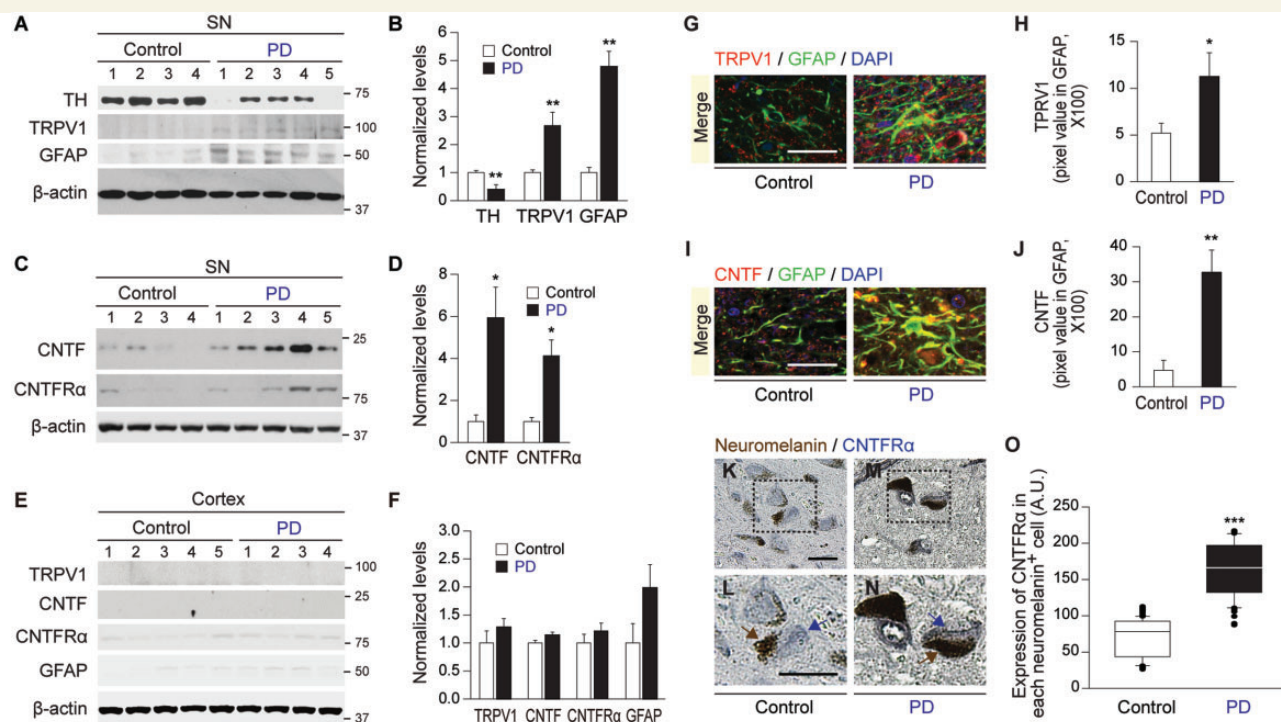
Figure 7 Expression of TH, TRPV1, GFAP, CNTF and CNTFRα in the substantia nigra of human Parkinson's disease brain. (A–F) Western blot analysis of TH, TRPV1, GFAP, CNTF, and CNTFRα in the SN (A–D) or cortex (E and F) of human control and Parkinson's disease brain. (G–J) Immunohistochemical analysis of TRPV1 (red, G) or CNTF (red, I) in GFAP$^+$ astrocytes (green, G and I) in the SNpc of human control or Parkinson's disease brain. DAPI (blue) stained nucleus. Quantification of TRPV1 (H) or CNTF (J) expression co-localized in GFAP$^+$ astrocytes in the SNpc of human brain, respectively (K–O) Photomicrographs of neuromelain$^+$ dopamine neurons (brown) and CNTFRα$^+$ cells (blue) in the SNpc of human control (K and L) and Parkinson's disease (M and N) brain. L and N show enlargement of areas depicted in the boxes of K and M, respectively. Quantification of CNTFRα expression in each neuromelanin$^+$ dopamine neuron in the SNpc of human brain (O). One hundred and fifty-six (control) or 65 (Parkinson's disease) neuromelanin$^+$ cells were analysed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from the corresponding control. Scale bars: G and I, 20 μm; K–M, 40 μm. Mean ± SEM; A–F, $n = 4$–5 in each group. G–O, $n = 3$ for TRPV1, CNTF and CNTFRα. ANOVA and Student-Newman-Keuls analysis.

expressed in astrocytes might function as endogenous neuroprotective machinery *in vivo* as MPP$^+$-induced neurotoxicity was more aggravated in the SNpc when TRPV1 was knocked down.

Our current results demonstrate that astrocytic TRPV1 mediates production of endogenous CNTF, which prevents MPP$^+$-induced degeneration of dopamine neurons *in vivo*. In addition, endogenous CNTF derived from astrocytes increased TH enzyme activity *in vivo* as assessed by pTHser31 suggesting that it not only protects against the loss of dopamine neurons, but it also contributes to behavioural recovery through increased TH enzymatic activity. Consistent with the notion that CNTF is neuroprotective is the observation that exogenous administration of CNTF prevents degeneration of nigral dopamine neurons in nigrostriatal pathway-transected rat (Hagg and Varon, 1993). Because TRPV1 and CNTF are significantly expressed in astrocytes in the SN of Parkinson's disease brain and of Parkinson's disease animal models, the present results suggest a path for pharmacologically promoting the utilization of endogenous CNTF in Parkinson's disease for neuroprotection via capsaicin activation of TRPV1

receptors. This approach has the promising potential to bypass the low CNS bioavailability and side effects of exogenously administered CNTF (Thoenen and Sendtner, 2002).

Neurotrophic factors, promising disease-modifying agents are commonly explored for use in the therapy of Parkinson's disease (Ramaswamy *et al.*, 2009; Aron and Klein, 2011). Although various neurotrophic factors have powerful protective restorative effects on dopamine neurons, the experimental and clinical data suggest that improved brain delivery of neurotrophic factors (sustained and localized neurotrophic factor delivery by crossing the blood–brain barrier) is crucial for producing beneficial results. One of the promising approaches to achieve this is to search for an endogenous system locally producing neurotrophic factors in brain and/or small molecules that can activate this endogenous system producing neurotrophic factors by passing through the blood–brain barrier. Furthermore, neurotrophic factor use is a safer and efficient gene therapy when expressed in astrocytes compared to its expression in neurons in MPTP and 6-OHDA models of Parkinson's disease (Drinkut *et al.*, 2012). Taken together,

astrocytic TRPV1-derived endogenous CNTF production by capsaicin described in our current data has the *in vivo* neuroprotective properties both in toxin (MPP$^+$)- or gene-(α-synuclein, *SNCA*) based models of Parkinson's disease, suggesting that this endogenous system producing CNTF in astrocytes can overcome the substantial problems for clinical application of neurotrophic factors and might be a promising therapeutic target for the treatment of Parkinson's disease.

In summary, the present work describes the beneficial effects of astrocytic TRPV1-derived endogenous CNTF (neuroprotection and stimulation of TH enzymatic activity) in the rat MPP$^+$ and α-synuclein models of Parkinson's disease. Thus, astrocytic TRPV1 activation by capsaicin or related compounds may have therapeutic potential for the treatment of Parkinson's disease to prevent the progressive degeneration of dopamine neurons and to enhance TH enzyme activity needed for functional recovery.

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Supplementary material

Supplementary material is available at *Brain* online.

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