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Research Paper

Integrin CD11b mediates α-synuclein-induced activation of NADPH oxidase through a Rho-dependent pathway

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ABSTRACT

The activation of microglial NADPH oxidase (NOX2) induced by α-synuclein has been implicated in Parkinson's disease (PD) and other synucleinopathies. However, how α-synuclein activates NOX2 remains unclear. Previous study revealed that both toll-like receptor 2 (TLR2) and integrin play important roles in α-synuclein-induced microglial activation. In this study, we found that blocking CD11b, the α chain of integrin $\alpha_M\beta_2$, but not TLR2 attenuated α-synuclein-induced NOX2 activation in microglia. The involvement of CD11b in α-synuclein-induced activation of NOX2 was further confirmed in $CD11b^{-/-}$ microglia by showing reduced membrane translocation of NOX2 cytosolic subunit $p47^{phox}$ and superoxide production. Mechanistically, α-synuclein bound to CD11b and subsequently activated Rho signaling pathway. α-Synuclein induced activation of RhoA and downstream ROCK but not Rac1 in a CD11b-dependent manner. Moreover, siRNA-mediated knockdown of RhoA impeded NOX2 activation in response to α-synuclein. Furthermore, we found that inhibition of NOX2 failed to interfere with the activation of RhoA signaling and interactions between α-synuclein and CD11b, further confirming that NOX2 was the downstream target of CD11b. Finally, we found that genetic deletion of CD11b abrogated α-synuclein-induced NOX2 activatoin *in vivo*. Taken together, our results indicated that integrin CD11b mediates α-synuclein-induced NOX2 activation through a RhoA-dependent pathway, providing not only a novel mechanistic insight but also a new potential therapeutic target for synucleinopathies.

1. Introduction

The synucleinopathies are a diverse group of neurodegenerative disorders including Parkinson's disease (PD), dementia with Lewy bodies, pure autonomic failure and multiple system atrophy [1,2]. Abnormal aggregates of α-synuclein (α-Syn) in selective populations of neuron and glia are the common pathological features shared by synucleinopathies [1,2]. In clinic, the therapeutic interventions for synucleinopathies are mainly target symptoms, which partially improves the life quality of patients, but does not alter disease progression [3]. The obscure of mechanisms greatly hampers the development of novel therapeutic strategies for synucleinopathies. Therefore, elucidating the potential mechanisms of synucleinopathies is urgently needed.

Strong evidence indicated that neuroinflammation is implicated in synucleinopathies, especially PD [4–6]. Activated microglia and accumulation of proinflammatory factors are present in the subsantia nigra (SN) and striatum of patients with PD [7,8]. Moreover, epidemiological and experimental animal studies demonstrated that non-steroidal anti-inflammatory drugs reduce the risk of acquiring PD [9–11]. Suppression of microglial activation also displays neuroprotection in animal models of multiple system atrophy [8]. α-Syn has been shown to be able to activate microglia and cause proinflammatory responses in the brain parenchyma, which exacerbates pathogenic processes [12]. NADPH oxidase (NOX2), a superoxide-producing enzyme, is subsequently

Abbreviations: Aβ, β-amyloid; DHE, dihydroethidium; DPI, diphenyliodonium; ERK, extracellular regulated protein kinases; HMGB1, high-mobility group box 1; Iba-1, ionized calcium binding adaptor molecule 1; LPS, lipopolysaccharide; Mac1, macrophage antigen complex-1; NOX2, NADPH oxidase; PD, Parkinson's disease; SN, subsantia nigra; SOD, superoxide dismutase; α-Syn, α-synuclein; TLR2, toll-like receptor 2

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recognized to be critical for α-Syn-induced microglial activation and neurodegeneration since pharmacological inhibition or genetic deletion of NOX2 attenuates α-Syn-induced microglial activation and related neurotoxicity [13,14]. However, how α-Syn induces NOX2 activation remains undefined.

Toll-like receptor 2 (TLR2) is recently identified as a novel receptor for α-Syn to trigger immune cell responses [12,15]. The TLR2 ligand activity of α-Syn seems to be highly conformation-selective and only certain types of oligomer can activate TLR2 [16]. In agreement, TLR2-independent effects of α-Syn on microglia were reported. Kim et al. demonstrated that not TLR2 but integrin is required for α-Syn-induced migration and morphological changes in microglia [17]. Similarly, integrin, especially macrophage antigen complex-1 (Mac1), was involved in microglial activation induced by A30P and A53T mutant α-Syn [18]. However, it is still unclear whether TLR2 or integrin contributes to α-Syn-induced activation of NOX2.

In the present study, we sought to identify the molecular mechanisms involved in α-Syn-induced NOX2 activation by using microglial cultures and transgenic mice. The potential roles of both TLR2 and integrin in α-Syn-induced activation of NOX2 were investigated. We found that not TLR2 but integrin CD11b, the α chain of $\alpha_M\beta_2$, was required for NOX2 activation induced by α-Syn in both *in vitro* and *in vivo*. The Rho signaling pathway was subsequently recognized to be critical for CD11b-mediated NOX2 activation.

2. Materials and methods

2.1. Reagents

The recombinant human α-Syn (S1001, endotoxin level, less than 0.024 EU/μg) was obtained from rPeptide (Bogart, GA, USA). HiLyte™ Fluor 488 labeled recombinant human α-Syn (1 - 140) (AS-55457) was purchased from AnaSpec, Inc (Fremont, CA, USA). Active Rho Pull-Down and Detection Kit (16116) and Active Rac1 Pull-Down and Detection Kit (16118) were purchased from Thermo Scientific (Rockford, lL, USA). The RhoA siRNA (sc-36414) was provided by Santa Cruz Biotechnology (Dallas, TX, USA). The Vectastain ABC Kit was provided by Vector Laboratories, Inc (PK-4001, Burlingame, CA, USA). The membrane protein extraction kit was obtained from Beyotime (P0033, Jiangsu, China). Diphenyliodonium (DPI, D2926) and RGD peptide (S8008) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and SelleckChemicals (Houston, TX, USA), respectively. The following primary antibodies were used: anti-$p47^{phox}$ (07–500, EMD Millipore Corporation, Billerica, MA, USA), anti-$gp91^{phox}$ (611414, BD Transduction Laboratories, San Jose, CA, USA), anti-CD11b (ab128797), anti-α-Synuclein (ab6162), anti-TLR2 (ab108998), anti-ROCK1 (ab97592), anti-GAPDH (ab181602, Abcam, Cambridge, MA, USA) and ionized calcium binding adaptor molecule-1 (Iba1; 019–19741, Wako Chemicals, Richmond, VA, USA), The BCA Protein Assay Kit was purchased from Life Technologies (#23250, Waltham, MA USA). All other chemicals were of the highest grade commercially available.

2.2. Primary microglial cultures

Primary microglial cells were prepared from whole brains of 1-d-old wild type (WT) or $CD11b^{-/-}$ mice pups as described previously [19]. Two weeks after seeding, microglia (purity of 95–98%) were shaken off for 30 min at 180 rpm at 37 °C. Microglia were seeded in 6 or 96-well plates overnight in Dulbecco's modified eagle medium/F12 supplemented with 10% FBS before treatment.

2.3. BV2 microglial cells

The mouse microglia BV2 cell line was maintained as described previously [20]. Briefly, BV2 microglial cells were maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified incubator with 5% CO_2 and 95% air. The cells were split or harvested every 3 − 5 days.

2.4. Preparation of α-Syn aggregates

The recombinant human α-Syn was dissolved in water at 1 mg/ml and then was incubated with agitation at 37 °C for 7 days to allow aggregation [13]. The concentrations of α-Syn aggregates were determined based on the initial concentration of α-Syn.

2.5. Measurement of superoxide

The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of WST-1 as described previously [21,22]. Briefly, microglial cells (1×10^5/well) were grown overnight in 96-well plates in DMEM/F12 medium containing 10% FBS and switched to phenol red–free HBSS (50 μl/well). Subsequently, 50 μl HBSS with and without SOD (50 U/ml) was added to each well along with 50 μl WST-1(1 mM) in HBSS and 50 μl vehicle or α-Syn. The absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices). The difference between the absorbance in the presence and absence of SOD was considered to be the amount of produced superoxide.

2.6. Co-immunoprecipitation

Co-immunoprecipitation was performed according to our previous report [23]. Briefly, recombinant human α-Syn was mixed with microglial lysates **(300 μg)** in IP lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 10 μl/ml proteinase inhibitor Cocktail). The lysate was centrifuged at 8200 × *g* for 20 mins at 4 °C. The supernatant was incubated at 4 °C with anti-α-Syn antibody **(10 μg)** or control IgG overnight and with protein A-Sepharose beads (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for additional 2 h. After washing, the beads were eluted and the samples were analyzed by Western blot for CD11b or α-Syn.

2.7. Confocal microscopy

To observe the surface binding of α-Syn, microglial cells were seeded in glass-bottom dishes and were treated with HiLyte™ Fluor 488 labeled recombinant human α–synuclein (200 nM) on ice for 20 mins according to a previous report [24]. After washing three times with cold PBS, cells were fixed with 4% paraformaldehyde and permeabilized by 0.05% Triton-X. Cells were then incubated with anti-CD11b antibody overnight at 4 oC, followed by adding Alexa 594-labeled secondary Ab (1:1000). The double-label immunofluorescence pictures were taken by the confocal microscope.

2.8. Rac1 and RhoA activation assays

The activation of Rac1 and RhoA was determined using active Rac1 and Rho pull-down and detection kit, respectively, according to the manufacturers' instruction. Briefly, microglial cells were lysized using 1X lysis/binding/wash buffer, and then centrifuged at 16,000 × *g* at 4 °C for 15 min. The active Rac1-GTP or RhoA-GTP was pulled down from 500 μg of supernatant using GST-PAK1-PBD (20 μg) or GST rhotekin-RBD (400 μg), respectively. For both assays, samples were incubated at 4 on a rotator for 1 h, washed three times in 1X lysis/binding/wash buffer, resuspended in reducing sample buffer, and then were analyzed by immunoblot analysis using antibody specifically against Rac1 or RhoA.

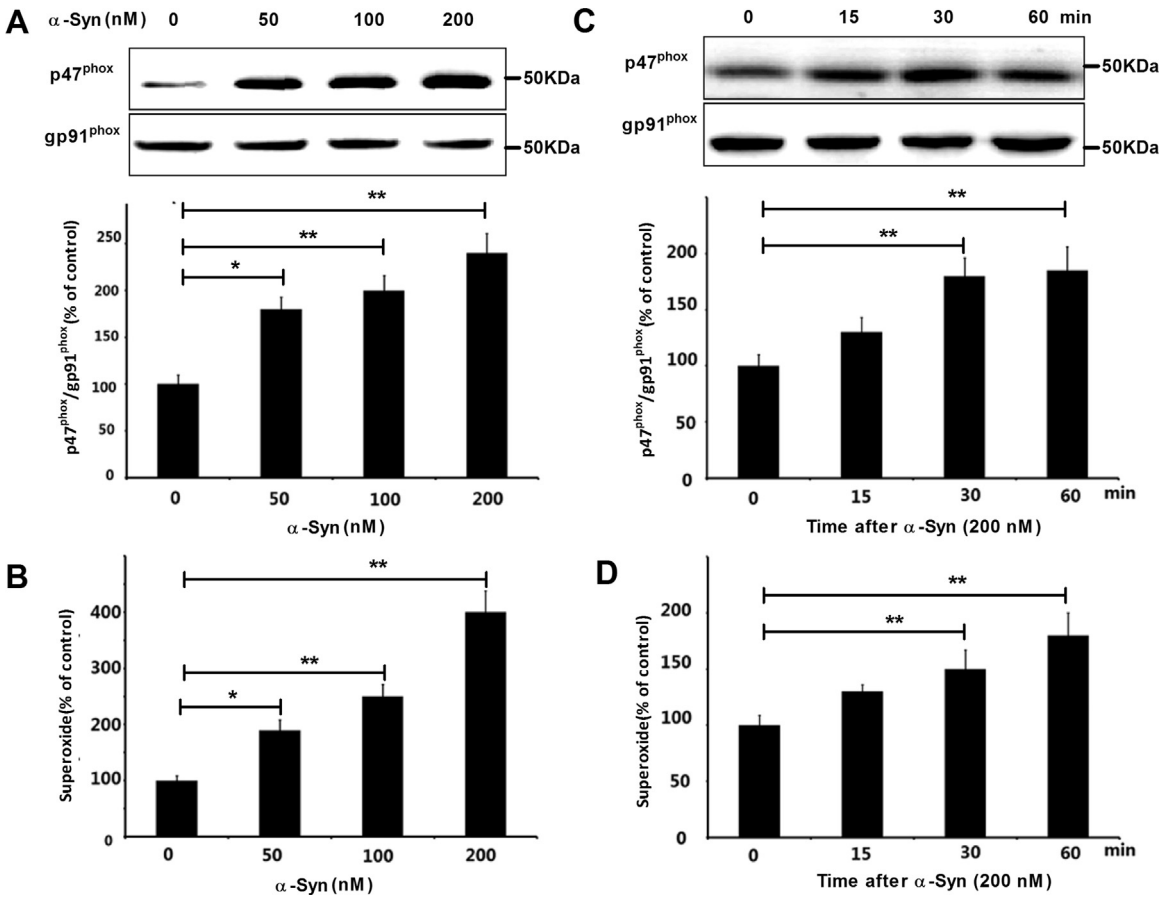

Fig. 1. α-Syn activates NOX2. (A) Primary microglial cells were treated with different concentrations of α-Syn. Sixty minutes later, the membrane translocation of $p47^{phox}$ was measured by using western blot and the density of blot was quantified. $Gp91^{phox}$ was used as an internal membrane control. (B) The dose-dependent production of superoxide in α-Syn-treated microglia was measured. (C) α-Syn-induced membrane translocation of $p47^{phox}$ was measured at the indicated time points after treatment by using western blot and the density of blot was quantified. (D) The time-dependent production of superoxide in α-Syn-treated microglia was measured. Results are expressed as mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

2.9. Knockdown of RhoA in microglia using siRNA

Microglial cells were seeded on 6 or 24-well plates and transfected by adding Opti-MEM reduced serum media containing a mixture of Lipofectamine RNAiMAX transfection reagent and ON-TARGETplus SMARTpool mouse RhoA-specific siRNA according to manufactures' instruction.

2.10. Animal treatments

Three month-old male WT (C57BL/6 J) and $CD11b^{-/-}$ mice were deeply anesthetized using chloral hydrate (300 mg/kg, s.c.) and then fixed on the stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). α-Syn (2 μg in a volume of 2 μl of PBS] was injected unilaterally to the SN. The other side was injected with PBS. Stereotaxic co-ordinates used were AP − 2.8 mm, ML − 1.3 mm, and DV- 4.5 mm, according to one previous report [25]. The injection rate was 0.2 μl/min for both α-Syn and PBS. The animals were sacrificed 1 h after α-Syn injection and midbrain tissues were dissected. Experiments were performed in accordance with the Animal Guideline of Dalian Medical University. Housing and breeding of animals were performed strictly with Dalian Medical University's Guide for the Care and Use of Laboratory Animals. All experimental protocols were approved by and in agreement with the Ethical Committee of Dalian Medical University.

2.11. Immunohistochemistry

Immunohistochemistry were performed as described previously [26]. Briefly, the free-floating sections (35 μm) encompassing the entire midbrain cultures were immunoblocked with 4–10% NGS and then incubated with rabbit antibody to Iba-1 (1: 5000) for 24 h at 4 °C. Antibody binding was visualized using a Vectastain ABC Kit (Vector Laboratories, Inc) and diaminobenzidine substrate. Images were recorded with a CCD camera and the MetaMorph software (Molecular Devices). The densities of Iba-1 immunostaining in the SN were measured using ImageJ software (National Institutes of Health) [27].

2.12. Membrane extraction

The membrane fractions of microglia and midbrain tissue were prepared using the membrane protein extraction kit as described previously [20]. Briefly, microglia and midbrain tissue were lysed in lysis buffer A provided by the kit and then subjected to Dounce homogenization (20–25 St, tight pestle A). The lysates were centrifuged at 700 × *g* for 10 mins; the supernatant was collected and centrifuged at 14,000 × *g* for 30 mins. The pellets were suspended using extraction buffer B and incubated for 20 mins. After centrifugation at 14,000 × *g* for 5 mins, the supernatant was used as membranous fraction.

2.13. Western blot

For western blot analysis, equal amounts of protein were separated by 4–12% Bis-Tris Nu-PAGE gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk and incubated with primary antibodies (1:1000) against $p47^{phox}$, $gp91^{phox}$, Rac1-GTP, total-Rac1, RhoA-GTP, total-RhoA, ROCK, GAPDH

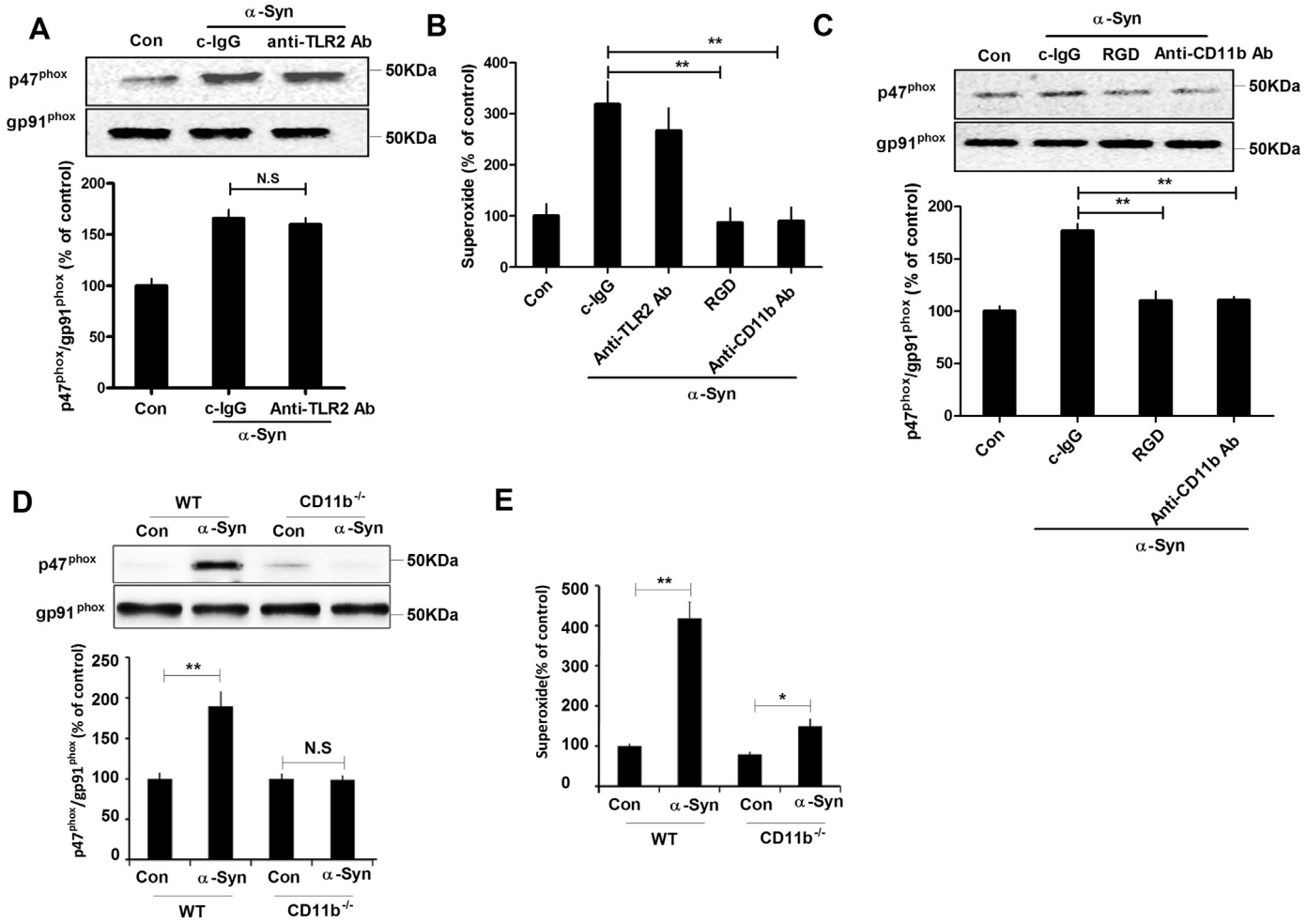

Fig. 2. Integrin CD11b but not TLR2 mediates α-Syn-induced activation of NOX2. (A) BV2 microglial cells were pre-treated with anti-TLR2 antibody (2.5 μg/ml) for 30 mins and then were treated with α-Syn for additional 60 mins. The membrane translocation of $p47^{phox}$ was determined in α-Syn-treated microglia with or without anti-TLR2 antibody by using western blot and the density of blot was quantified. (B) The production of superoxide was measured in α-Syn-treated microglia with or without anti-TLR2 antibody, RGD (50 μM) or anti-CD11b antibody (2.5 μg/ml) pre-treatment for 30 mins. (C) The membrane translocation of $p47^{phox}$ was measured in α-Syn-treated microglia with or without RGD or anti-CD11b antibody by using western blot and the density of blot was quantified. (D) The membrane translocation of $p47^{phox}$ was measured in α-Syn-treated WT and $CD11b^{-/-}$ primary microglia by using western blot and the density of blot was quantified. (E) The production of superoxide was measured in α-Syn-treated WT and $CD11b^{-/-}$ microglia. Results are expressed as mean ± SEM from three independent experiments. N.S, not significant; c-IgG: control IgG. $*p < 0.05$, $**p < 0.01$.

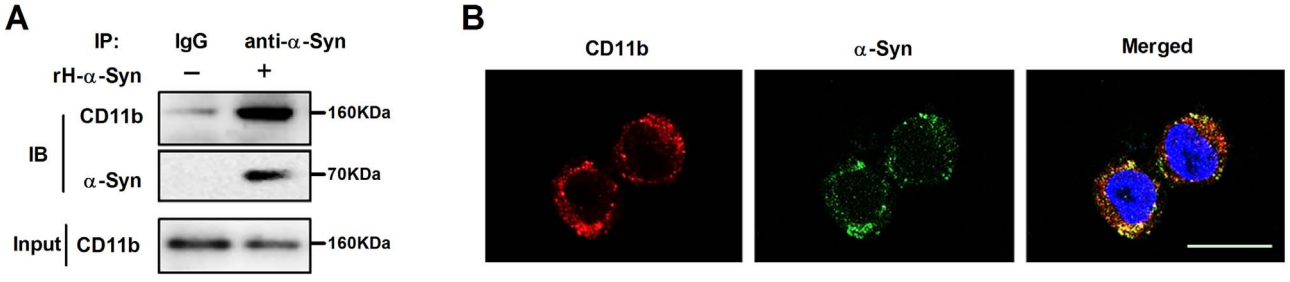

Fig. 3. α-Syn binds to CD11b. (A) Purified recombinant human α-Syn aggregates were mixed with microglial lysates to allow α-Syn to react with CD11b, and then the mixtures were immunoprecipitated with antibody against α-Syn. Binding was detected via immunoblot for CD11b and α-Syn. (B) Confocal microscopy analysis further delineated the interaction between α-Syn and CD11b on the cell surface. This experiment was performed 4 independent times with duplication. Bar = 10 μm.

and HRP-linked anti-rabbit or mouse IgG (1:3000) for 2 h. ECL reagents were used as a detection system.

2.14. Statistical analysis

All values are expressed as the mean ± SEM. Differences among means were analyzed using one- or two-way ANOVA with treatment as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls post hoc testing. In all analyses, a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Blocking integrin but not TLR2 attenuates α-Syn-induced NOX2 activation in microglia

To investigate whether TLR2 or integrin is involved in α-Syn-

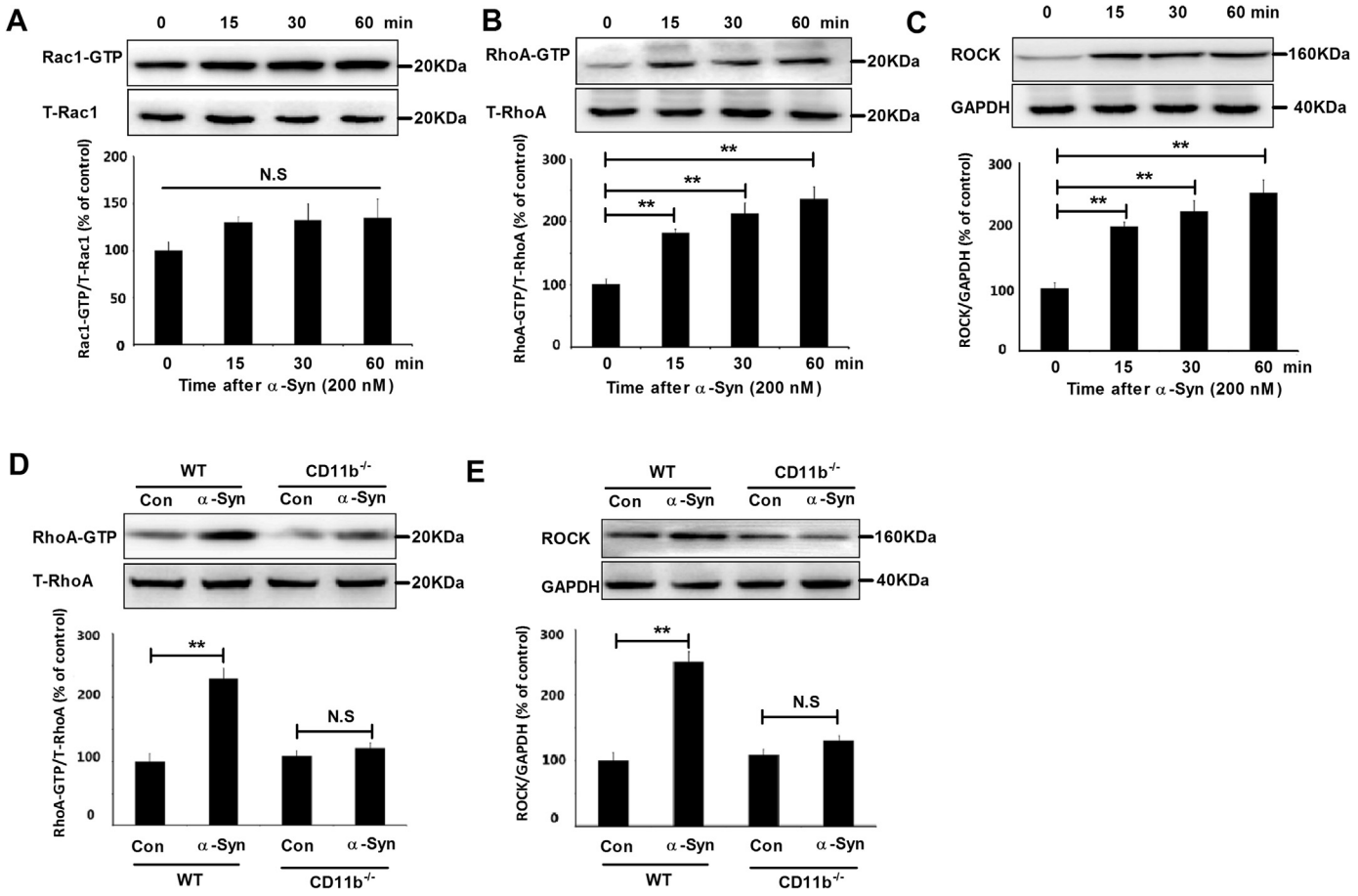

Fig. 4. α-Syn stimulates activation of RhoA pathway in a CD11b-dependent manner. (A–C) Microglia were treated with α-Syn for 15, 30 or 60 mins. The levels of Rac1-GTP, total-Rac1 (A), RhoA-GTP, total RhoA (B) and ROCK (C) were determined by western blot using specific antibodies. The density of blot was quantified. (D, E) Microglia prepared from WT and CD11b$^{-/-}$ mice were treated with α-Syn for 60 mins. Levels of RhoA-GTP, total-RhoA (D) and ROCK (E) were determined by western blot using specific antibodies and the density of blot was quantified. GAPDH was used as an internal control. Data are expressed as mean ± SEM from three independent experiments. N.S, not significant; $^{**}p < 0.01$.

induced NOX2 activation, we initially examined the stimulatory effects of α-Syn on NOX2 in our system. Primary microglia were treated with the indicated concentrations of α-Syn and the membrane translocation of NOX2 cytosolic subunit p47^{phox}, an essential step for NOX2 activation, was detected. As shown in Fig. 1**A**, compared the vehicle control, the levels of p47^{phox} in membrane fractions prepared from 50, 100 and 200 nM α-Syn-treated microglia were gradually increased, indicating dose-dependent activation of NOX2. α-Syn-induced NOX2 activation was further confirmed by measuring the production of superoxide. A dose-dependent pattern for α-Syn-induced superoxide production was observed in microglia (Fig. 1**B**). Time course study revealed that α-Syn-induced p47^{phox} membrane translocation and superoxide production also followed a time-dependent manner and peaked at 60 mins after treatment (Fig. 1**C and D**).

Next, we determined the role of TLR2 and integrin in α-Syn-induced NOX2 activation. The activity of TLR2 in microglia was blocked using anti-TLR2 antibody. As seen in Fig. 2**A**, blocking TLR2 failed to interfere with α-Syn-induced membrane translocation of p47^{phox}, suggesting that TLR2 is not required for α-Syn-induced NOX2 activation. This conclusion was further confirmed by showing that anti-TLR2 antibody failed to attenuate superoxide production in α-Syn-treated microglia (Fig. 2**B**). In contrast, blocking integrin using RGD peptide, a cell adhesion motif that can bind to integrin, markedly mitigated α-Syn-induced p47^{phox} membrane translocation and superoxide production in microglia (Fig. 2**B and C**), suggesting that integrin is necessary for α-Syn-induced activation of NOX2.

3.2. CD11b integrin is necessary for α-Syn-induced NOX2 activation

CD11b is the α-chain of $\alpha_M\beta_2$ (also called CD11b/CD18) integrin and presents with high level in innate immune cells, including microglia [28]. To determine whether integrin CD11 is involved in α-Syn-induced NOX2 activation, anti-CD11b antibody was used to block CD11b activation. As shown in Fig. 2**B and C**, blocking CD11b significantly suppressed p47^{phox} membrane translocation and production of superoxide in microglia treated with α-Syn.

To further confirm the involvement of CD11b in α-Syn-induced NOX2 activation, microglial cells deficient in CD11b were prepared. We found that p47^{phox} membrane translocation was detected in α-Syn-treated WT but not CD11b$^{-/-}$ microglia (Fig. 2**D**). In agreement, microglia deficient in CD11b were more resistant to α-Syn-induced superoxide production than WT controls (Fig. 2**E**).

3.3. α-Syn binds to CD11b

To investigate the mechanisms by which CD11b mediates α-Syn-induced NOX2 activation, the interaction of α-Syn and CD11b was determined. Recombinant human α-Syn was used to coimmunoprecipitate CD11b. As shown in Fig. 3**A**, α-Syn pulled down CD11b from membrane extracts prepared from microglia. Reciprocally, CD11b was also able to coprecipitate α-Syn, whereas neither CD11b nor α-Syn was pulled down by control IgG. To further confirm the interactions between α-Syn and CD11b, a binding assay was performed. Immunofluorescence staining showed a surface binding of Fluro 488 labeled α-Syn (green) in microglia. Co-staining with anti-CD11b antibody

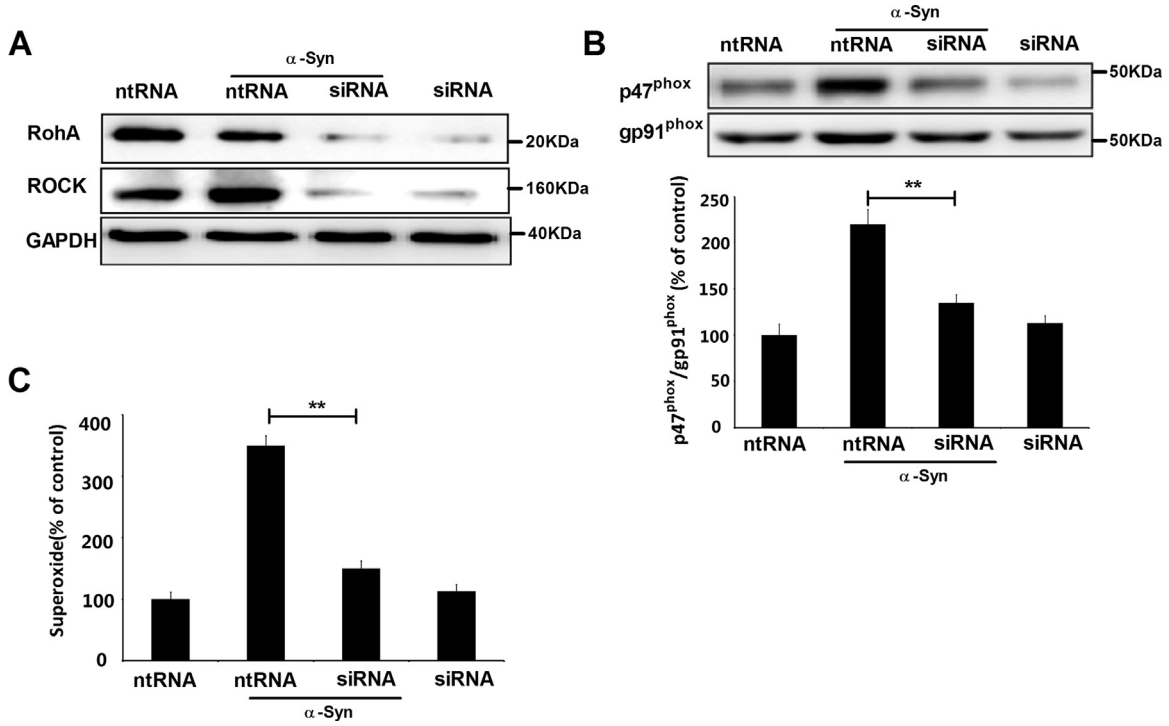

Fig. 5. Knockdown of RhoA impairs α-Syn-induced NOX2 activation. (A) RhoA-specific or control siRNA was transfected to microglia and the efficacy was shown. (B) α-Syn-induced membrane translocation of $p47^{phox}$ was measured in microglia transfected with RhoA-specific or control siRNA by using western blot and the density of blot was quantified. (C) The effects of RhoA knockdown on α-Syn-induced superoxide production were determined. Results are expressed as mean ± SEM from three independent experiments. $^{**}p < 0.01$.

revealed colocalization of CD11b (red) with surface-bound α-Syn (Fig. 3**B**), indicating a direct binding between CD11b and α-Syn.

3.4. α-Syn induces activation of Rho signaling pathway through a CD11b-dependent manner

We next explored the downstream events occurring after α-Syn binds to CD11b. It has been documented that Rho family GTPases are essentially involved in superoxide formation during phagocytosis of immune cells [29]. We therefore investigated the effects of α-Syn on the activation of Rac1 and RhoA, two widely studied members of Rho family. Results showed that compared with vehicle controls, the activation of RhoA was significantly increased in α-Syn-treated microglia by showing elevated expressions of active RhoA (RhoA-GTP) in a time-dependent manner, although the levels of active Rac1 (Rac1-GTP) were unchanged (Fig. 4**A and B**). Consistent with RhoA activation, the expression of ROCK, a downstream effector of Rho kinase, was also time-dependently increased in α-Syn-treated microglia (Fig. 4**C**).

To determine whether CD11b mediates α-Syn-induced activation of RhoA signaling, the activation of RhoA and ROCK was detected in $CD11b^{-/-}$ microglia. As seen in Fig. 4**D and E**, α-Syn elevated the levels of both RhoA-GTP and ROCK in WT microglia, which was significantly reduced in microglia deficient in CD11b, suggesting that CD11b contributes to α-Syn-induced activation of RhoA signaling.

3.5. RhoA knockdown blocks α-Syn-induced activation of NOX2

To investigate whether Rho signaling is involved in α-Syn-induced NOX2 activation, RhoA was silenced by using siRNA. The efficiency of siRNA was initially examined. As seen in Fig. 5**A**, the expressions of both RhoA and ROCK were markedly reduced in microglia transfected with RhoA siRNA, but not control siRAN. In agreement with inhibition of Rho signaling, the membrane translocation of $p47^{phox}$ and superoxide production induced by α-Syn were greatly impeded in RhoA siRNA-transfected microglia (Fig. 5**B and C**), suggesting that Rho signaling mediates α-Syn-induced NOX2 activation. However, transfection with control siRNA failed to interfere with α-Syn-induced NOX2 activation.

3.6. Inhibition of NOX2 fails to interfere with activation of CD11b

The activation of integrin can be regulated by both outside-in and inside-out signals [30]. We next determined whether NOX2-derived superoxide could be served as a paracrine signal for CD11b activation. Since Rho pathway is also involved in regulating CD11b activation by inside-out signals [31], the effects of NOX2 on α-Syn-induced activation of Rho signaling were initially determined. The activity of NOX2 was blocked by DPI, a widely used NOX2 inhibitor. No significant difference of α-Syn-induced Rho signaling activation was observed in microglia with or without DPI treatment as shown by a comparable level of RhoA-GTP and ROCK between combined DPI and α-Syn and α-Syn alone group (Fig. 6**A and B**).

Next, the expression of CD11b in α-Syn-treated microglia was measured. As seen in Fig. 6**C**, inactivation of NOX2 by DPI or down-regulation of Rac1 or RhoA by siRNAs had no effect on CD11b expression in microglia treated with α-Syn. Inside-out signaling leading to integrin activation can also be measured by binding of ligands with integrin [28]. Therefore, the effects of NOX2 and Rho signaling on interactions between α-Syn and CD11b were determined. In agreement with no alteration of CD11b expression, both DPI and RhoA siRNA also failed to interfere with the interactions between α-Syn and CD11b (Fig. 6**D and E**). These results suggest that NOX2 is a passive downstream target of integrin CD11b.

3.7. CD11b mediates α-Syn-induced NOX2 activation in vivo

To determine whether CD11b mediates α-Syn-induced activation of NOX2 *in vivo*, α-Syn was injected stereotaxically into the SN of both WT and CD11b-deficient mice (Fig. 7**A**). The activation of NOX2 was examined by detecting the membrane translocation of $p47^{phox}$. Consistent

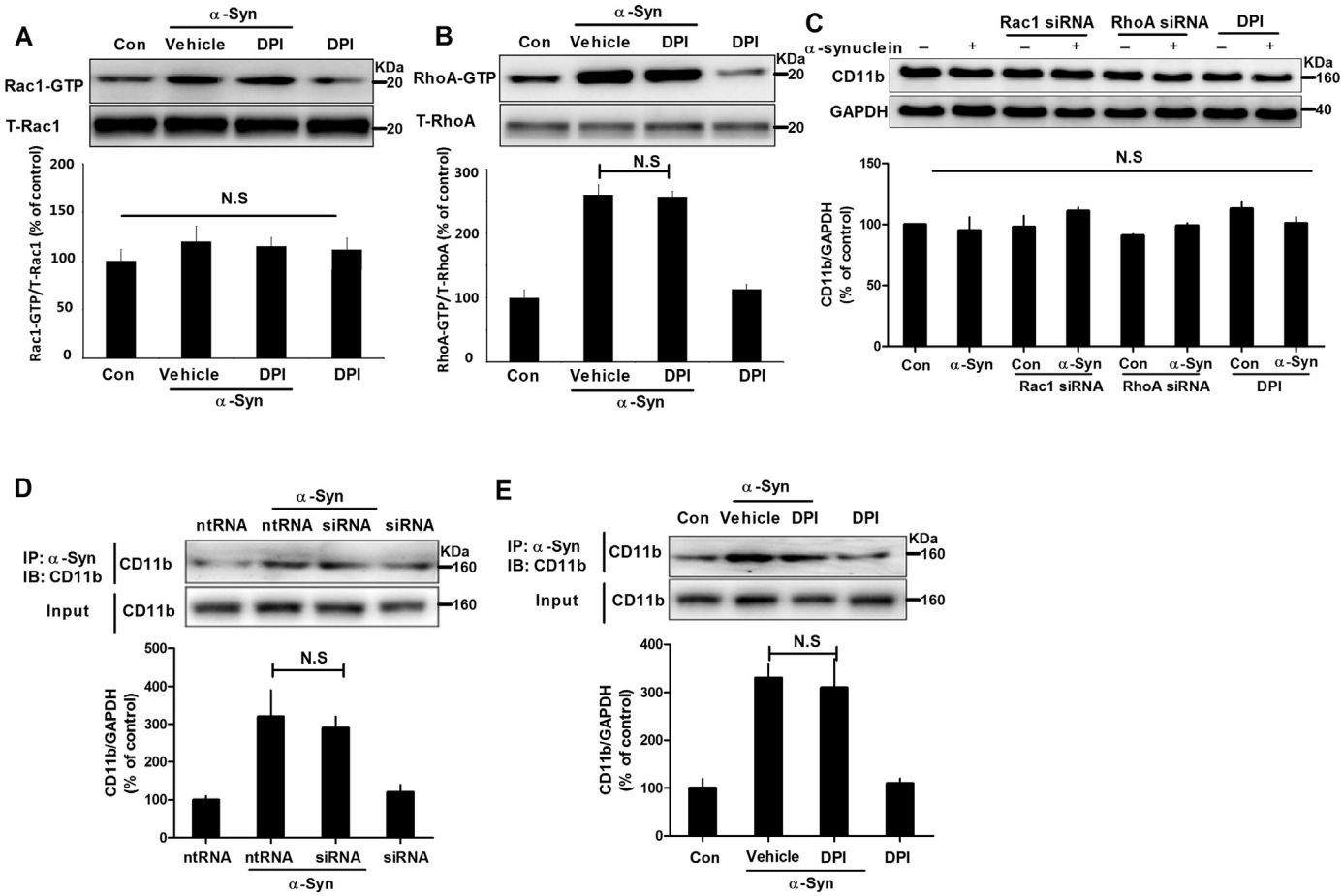

Fig. 6. Blocking NOX2 fails to affect activation of RhoA pathway and CD11b. (A) α-Syn-induced activation of RhoA was determined in microglia pre-treated with NOX2 inhibitor, DPI (10 μM) for 30 mins by using western blot and the density of blot was quantified. DPI at this concentration displays potent inhibitory effects on NOX2 activation and superoxide production in our previous study [21]. (B) The effects of DPI on α-Syn-induced expression of ROCK were determined by using western blot and the density of blot was quantified. (C) α-Syn-induced CD11b expression was determined in microglia transfected with Rac1-specific, RhoA-specific or control siRNA or pre-treated with DPI by using western blot. The density of blot was quantified. (D, E) The effects of RhoA knockdown (D) or NOX2 inhibition (E) on the interactions of α-Syn and CD11b were determined by co-immunoprecipitation. Results are expressed as mean ± SEM from three independent experiments. N.S, not significant.

with *in vitro*, the levels of $p47^{phox}$ in the membrane fractions were significantly increased in α-Syn-injected WT mice (Fig. 7**B**), suggesting that α-Syn activates NOX2 *in vivo*. However, mice deficient in CD11b displayed resistance for α-Syn-induced activation of NOX2 since no significant membrane translocation of $p47^{phox}$ was detected in midbrain tissues prepared from $CD11b^{-/-}$ mice (Fig. 7**B**). NOX2 activation was associated with activation of microglia in α-Syn-treated WT mice as shown by elevated Iba-1 expression and enlarged cell body size compared with vehicle controls (Fig. 7**C and D**). In agreement with inactivation of NOX2, activated microglia were not detected in $CD11b^{-/-}$ mice injected with α-Syn (Fig. 7**C and D**).

4. Discussion

In this study, we demonstrated that not TLR2 but integrin CD11b was required for α-Syn-induced activation of NOX2. For the mechanism of regulation, we discovered that α-Syn bound to CD11b and subsequently stimulated activation Rho signaling pathway, which triggered membrane translocation of NOX2 cytosolic subunit $p47^{phox}$, resulting in NOX2 activation. Finally, mice deficient in CD11b were more resistant to α-Syn-induced NOX2 activation than WT controls.

TLR2 is a key innate immune receptor belongs to pattern recognition receptors [32]. TLR2 was recently identified as an endogenous receptor for α-Syn [16]. However, studies delineating the role of TLR2 in α-Syn-mediated microglial activation have generated contrasting results. Kim et al. reported that neuron-released α-Syn stimulated activation of microglia and release of TNFα and IL-6 through a TLR2-dependent manner [16]. Similarly, TLR2-dependent microglial activation in α-Syn-treated microglial culture was also observed in Daniele et al.' study [12]. In contrast, TLR2-independent microglial activation in response to α-Syn was also reported in previous studies [17,18]. In this study, we found that blocking TLR2 failed to mitigate recombinant human α-Syn-induced NOX2 activation, supporting the TLR2-independent activity of α-Syn. Although the exact mechanism for both TLR2-dependent and -independent effects of α-Syn remains unknown, the intrinsic difference between recombinant and endogenous α-Syn might be one of the reasons. Additionally, the different structures of α-Syn used in these studies might also take a responsibility because the TLR2 ligand activity of α-Syn is conformation-sensitive; only specific types of oligomer can interact with and activate TLR2 [16].

The integrins are non-covalently-associated heterodimeric transmembrane receptors consisting of α and β chains [28]. CD11b, the α-chain of $\alpha_M\beta_2$, is highly expressed in innate immune cells and has been implicated in various immune cell response, such as adhesion, migration, phagocytosis, and chemotaxis [28]. It is both an adhesion molecule and a pattern recognition receptor. Multiple pathogen and damage-associated molecules such as endotoxin lipopolysaccharide (LPS) [33], PolyI:C [24], high mobility group box-1 protein (HMGB1) [34] and aggregated β-amyloid (Aβ) [35] can bind to CD11b. Previous study demonstrated that CD11b/CD18 is involved in adhesion-elicited NOX2 activation [36]. In the present study, we provided direct experimental evidence implicating the involvement of CD11b in α-Syn-stimulated

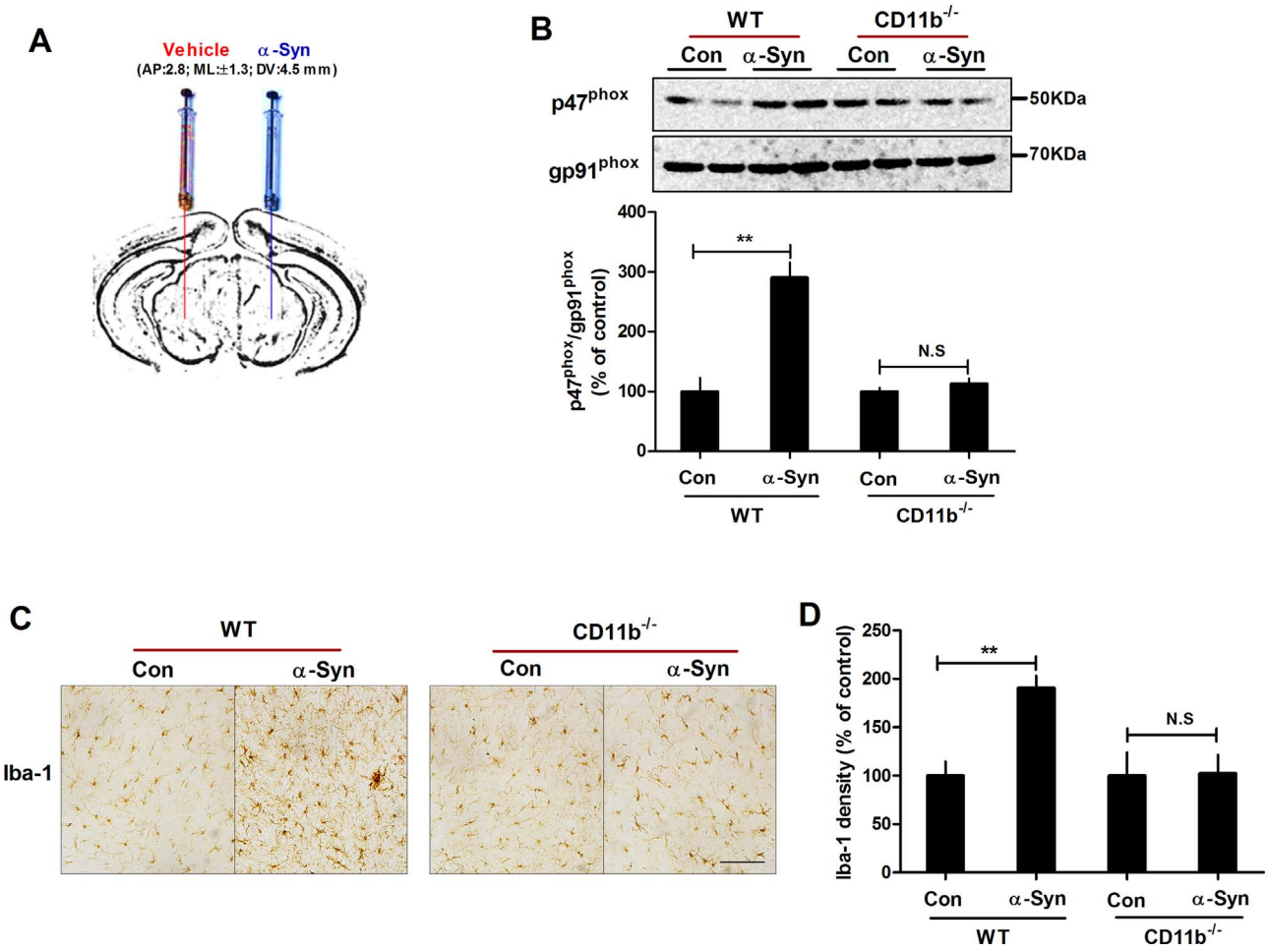

Fig. 7. CD11b deficiency attenuates α-Syn-induced NOX2 activation and microglial activation *in vivo*. (A) α-Syn or vehicle was injected into the SN of WT and CD11b$^{-/-}$ mice. (B) The midbrain tissues of mice were dissected and the membrane translocation of p47^{phox} was measured by using western blot. The density of blot was quantified. (C) Microglia in the SN were stained with anti-Iba-1 antibody and the representative images were shown. Activated microglia displayed enlarged cell body size and high staining density. (D) The density of Iba-1 staining in the SN was quantified **(6 sections each mouse)**. Results are expressed as mean ± SEM. n = 3; N.S, not significant; $^{**}p < 0.01$.

NOX2 activation. This conclusion is supported by the following experimental findings: first, α-Syn bound to CD11b; second, inhibition or genetic deletion of CD11b abrogated α-Syn-induced p47^{phox} membrane translocation and superoxide production; third, mice deficient in CD11b were more resistant to α-Syn-induced NOX2 activation. Consistent with our findings, the involvement of CD11b in NOX2 activation was also observed in HMGB1 and LPS-treated microglial cultures [34,37]. Taken together, these results lend strong credence to the concept that CD11b mediates α-Syn-stimulated activation of NOX2.

Mechanistically, the most critical question to address is how CD11b mediates α-Syn-elicited NOX2 activation. The Rho family of GTPases is one of the downstream signaling pathways of integrins and plays an important role in many diverse critical cellular processes, such as gene transcription, cell–cell adhesion, cell cycle progression and cell survival and death [29]. Although RhoA/ROCK is involved in superoxide formation [38], it is still remains unclear whether Rho signaling pathway is involved in α-Syn-induced activation of NOX2. Here, we found that α-Syn stimulated activation of RhoA and downstream ROCK, which was markedly mitigated by genetic deletion of CD11b. Moreover, silencing RhoA suppressed α-Syn-induced p47^{phox} membrane translocation and superoxide production. These results suggest that α-Syn-CD11b axis regulates NOX2 activation through a Rho signaling-dependent manner. Similarly, the activation of Rho signaling pathway was involved in angiotensin II-stimulated NOX2 activation [39]. Moreover, Rho signaling pathway-mediated NOX2 activation also contributed to hydrogen peroxide-induced vascular contraction [40]. The mechanism responsible for Rho signaling-mediated NOX2 activation might be related to the activation of extracellular signal-regulated kinase (ERK), a Rho downstream kinase that can phosphorylate p47^{phox} and subsequently result in membrane translocation [41] since α-Syn is well-known to be able to active ERK [42].

Integrin signals bidirectionally through the plasma membrane in pathways referred to as “inside-out” and “outside-in” signaling. Previous study indicated that Rho signaling might be one of the “inside-out” signals to regulate integrin. GM-CSF was found to regulate integrin through Rho GTPase pathway, leading to migration of human eosinophil [43]. Similarly, in Arita et al.'s study, silencing Rho/Rho-kinase, ROCK interfered with the expression of CD11b/CD18 in TNFα-mediated diabetic microvascular damage [44]. However, in the present study, silence of Rho signaling failed to affect both the expression of CD11b and interactions between α-Syn and CD11b. Although the underlying mechanisms remain unclear, lack of additional stimulating signal for the activation of Rho signaling might be one of the reasons. This speculation was supported by the findings that NOX2-derived superoxide failed to be as a paracrine signal to regulate α-Syn-induced RhoA activation, although inhibition of NOX2 blocked angiotensin II-induced activation of Rho signaling in previous report [45].

In conclusion, our studies reveal a new regulatory function of CD11b in α-Syn-induced NOX2 activation. We also identified Rho signal as a potential novel signaling pathway that bridges α-Syn-CD11b axis and subsequent NOX2 activation. These results help to expand our understanding for the pathogenesis of synucleinopathies. Our findings

also suggest that integrin, especially CD11b, may be a promising target for the treatment of synucleinopathies.

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Conflict of interest

The authors declare no conflict of interest.

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