Neurobiology of Disease 129 (2019) 1–12

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Nicotine prevents alpha-synuclein accumulation in mouse and human iPSC-derived dopaminergic neurons through activation of the dopamine D3-acetylcholine nicotinic receptor heteromer

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ARTICLE INFO

Keywords:
Alpha-synuclein
Ubiquitin-proteasome system (UPS)
Nicotine
Dopamine D3 receptor
Heteromerization
Nicotinic receptor
Human induced pluripotent stem cell (hiPSC)
Neuroprotection
Proximity Ligation Assay (PLA)

ABSTRACT

We recently found that in mouse dopaminergic neurons, the heteromer formed by the dopamine D3 receptor (D3R) and the β2 subunit of acetylcholine nicotinic receptor (nAChR) exerts neurotrophic effects when activated by nicotine, leading to neurons with enlarged cell bodies and increased dendrite arborization. Beside this action, we now show that nicotine, by activating the D3R-nAChR heteromer, protects dopaminergic neurons against neuronal injury. In primary cultures of mouse dopaminergic neurons, in fact, the ability of nicotine to inhibit both the pathological accumulation of alpha-synuclein induced by glucose deprivation and the consequent morphological defects were strongly prevented by disrupting the D3R-nAChR heteromer with specific interfering TAT-peptides; the relevance of the phosphoinositide 3-kinase (PI3K) intracellular signaling in mediating nicotine prevention of alpha-synuclein aggregation has been also demonstrated. Moreover, the ability of nicotine in restoring the ubiquitin-proteasome system has been found as a mechanism contributing to the neuroprotective properties of nicotine.

By using the proximity ligation assay, we have shown that the D3R-nAChR heteromer is also expressed in human dopaminergic neurons derived from induced pluripotent stem cells. In this human cell model, nicotine exerts neuroprotective effects specifically acting through the D3R-nAChR complex thus indicating that this heteromer is a relevant molecular effector involved in the protection of human dopaminergic neurons.

1. Introduction

The progressive degeneration of nigro-striatal dopaminergic (DA) neurons results in the severe motor deficits characteristic of Parkinson's disease (PD) (Lozano et al., 1998). The current therapy of PD improves the symptoms without affecting disease evolution; therefore, drugs protecting DA neurons against toxic insults and supporting neuronal restoration are needed to counteract disease progression.

DA neuron function is regulated by different neurotransmitter systems including dopamine (DA) itself and acetylcholine (ACh). It is well known that DA D2 receptors (D2R) and ACh nicotinic receptors (nAChR) containing the β2 subunit (β2-nAChR) located in DA nerve terminals control neuronal firing and DA release in opposite ways (Champtiaux et al., 2003; de Kloet et al., 2015; Quarta et al., 2007; Sokoloff et al., 2006). On the other hand, increasing evidence suggests that both D2-like agonists and nicotine, by increasing dendrite arborization and soma size, provide neurotrophic support to DA neurons (Bono et al., 2018; Collo et al., 2008; Du et al., 2005; Joyce and Millan, 2007; Van Kampen and Eckman, 2006). These effects are mediated by the D3 receptor (D3R) and the β2-nAChR expressed in somatodendritic compartments (Champtiaux et al., 2003; Diaz et al., 2000) and, by increasing synaptic connections among neurons, may be involved in the mechanisms supporting the strength of nigro-striatal DA transmission. Stimulation of both D3R and nAChR also provides neuroprotective effects to DA neurons in models of glucose deprivation (GD)-induced neuronal injury (Bellucci et al., 2008; Bono et al., 2018), suggesting

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https://doi.org/10.1016/j.nbd.2019.04.017
Received 23 January 2019; Received in revised form 5 April 2019; Accepted 29 April 2019
Available online 30 April 2019
0969-9961/

that these receptors also promote DA neuron survival. Removing excitatory cholinergic inputs to nigral DA neurons of rats and macaques by stereotaxic lesioning of the peduncolopontine cholinergic nucleus, results, in fact, in a significant DA neuron loss (Bensaid et al., 2016). On this line, an inverse correlation has been established between cigarette smoking and PD development (Fratiglioni and Wang, 2000; Picciotto and Zoli, 2008).

The structural remodeling of DA neurons elicited by nicotine has been shown to be strongly dependent on functional D3R since it was lost in DA neurons from D3R-KO mice and was prevented by D2R/D3R antagonists (Bono et al., 2018; Collo et al., 2008; Collo et al., 2013), suggesting a functional cross-talk between these receptor systems. On this line, we recently reported that in mouse DA neurons the β2 subunit of nAChR directly interacts with the D3R to form a heteromeric complex that represents the molecular unit underlying nicotine-mediated neurotrophic effects (Bontempi et al., 2017). This finding is in line with previous data suggesting that, as the majority of G protein-coupled receptors (GPCR), DA receptors form heteromers not only with other GPCR (Missale et al., 2010), but also with ion channel receptors including the glutamate NMDAR (Cahill et al., 2014; Fiorentini et al., 2003; Lee et al., 2002) and the GABA-AR (Liu et al., 2000).

The major neuropathological hallmark of PD is the presence of Lewy Bodies (LB), mainly containing aggregated alpha-synuclein (alpha-syn) (Longhena et al., 2018; Spillantini et al., 1998), a protein involved in the control of DA synaptic function (Abeliovich et al., 2000; Burré et al., 2015; Scott and Roy, 2012; Vargas et al., 2014). A causative link between alpha-syn pathological changes, DA neuron degeneration and the onset of PD has been proposed (Bellucci et al., 2017; Goedert et al., 2017; Spillantini and Goedert, 2018). Understanding the molecular mechanisms underlying alpha-syn function and dysfunction is thus crucial for better comprehending the pathophysiology of PD. Evidence that both D3R agonists and nicotine could modulate alpha-syn accumulation has been provided by using GD-induced neurotoxicity (Bellucci et al., 2008; Bono et al., 2018). In particular, exposure of both mouse and human induced pluripotent stem cell (hiPSC)-derived DA neurons to GD results in the pathological accumulation of alpha-syn, an effect prevented by both nicotine and D2R/D3R agonists (Bellucci et al., 2008; Bono et al., 2018). By using hiPSC-derived DA neurons, we recently reported that nicotine-induced inhibition of alpha-syn accumulation was blocked by D3R antagonists (Bono et al., 2018), suggesting that, as reported for the neurotrophic effects, neuroprotection and inhibition of alpha-syn accumulation in DA neurons likely requires a cross-talk between the nAChR and the D3R.

On this basis, the aim of this study was to investigate whether the D3R-nAChR complex plays a role in the modulation of GD-induced alpha-syn accumulation and related neuronal injury in both mouse and iPSC-derived human DA neurons.

2. Material and methods

2.1. Animals

D3R knock out mice (D3R-KO) were obtained from Jackson Laboratory (Bar Harbor, ME) (B6.129S4-Drd3Tm1Dac/J). C57BL6/J syngenic mice were used as control. Animals were bred and housed in the animal-house facility of the University of Brescia with water and food ad libitum and a 12-h light-dark cycle. Animals were used in accordance with the 2010/63/EU Directive. All procedures were conformed to the National Research Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Ethical Committee of the University of Brescia. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. D3R-nAChR complex interfering TAT-peptides

Cell-permeable peptides interfering with the interaction of D3R with the β2 subunit of the nAChR were obtained and characterized as previously described (Bontempi et al., 2017; Matera et al., 2019). Briefly, a 11 amino acid sequence of human immunodeficiency virus TAT transporter was linked to either the 215–225 arginine-rich region of D3R (TAT-D3R; NH_2 -YGRKKRRQRRR**LKQRRRKRIL**-COOH) or the 439–449 aspartate-rich region of β2 nAChR subunit (TAT-β2; NH_2-YGRKKRRQRRR**HMRSEDDDQSVS**-COOH) (GenScript, Piscataway, USA). Peptides with scrambled sequences were used as negative controls (TAT-D3R-Sc: NH_2-YGRKKRRQRRR**IRKLRLRQRK**-COOH; TAT-β2-Sc: NH_2-YGRKKRRQRRR**QMVDSDRHSSDE**-COOH).

2.3. Primary cultures of mouse midbrain neurons and treatments

Primary cultures of midbrain neurons were prepared from both wild-type and D3R-KO mice, as previously described (Bontempi et al., 2017). Briefly, the ventral mesencephalon was dissected from E12.5 mouse embryos, mechanically dissociated at room temperature, and suspended in Neurobasal medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 2 mM glutamine and 2% B27 supplement (Gibco, Invitrogen). Cells were seeded on poly-D-lysine/laminin-coated coverslips (8×10^4 cells/well) and cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Half of the medium was changed every 2 days until treatment. Ten days after seeding, wild-type and D3R-KO neuronal cultures were incubated in Hank's balanced salt solution (HBSS, Euroclone) supplemented with 2 mM glutamine and 2% B27 without glucose for 60 min at 37 °C (glucose deprivation, GD). Untreated neurons were maintained in supplemented Neurobasal medium. Neurons from wild-type mice were then incubated with nicotine (10 μM) for 48 or 120 h. Cells were fixed and analyzed by immunocytochemistry and immunofluorescence for both alpha-syn accumulation, thioflavin S-positive staining and morphology. In addition, wild-type neurons exposed to GD for 1 h were incubated with nicotine (10 μM) for 48 or 120 h in the presence or in the absence of both TAT-D3R (1 μM) and TAT-β2 (1 μM) peptides. In parallel, treatment with nicotine was performed in the presence of the scrambled peptides TAT-D3R-sc and TAT-β2-sc (both at 1 μM). Cells were fixed and analyzed for both alpha-syn accumulation and morphology. In some experiments, wild-type cultures were exposed to GD for 1 h and then to nicotine (10 μM; 48 h) in the presence or in the absence of the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (10 μM), added 30 min before nicotine treatment, and analyzed for alpha-syn accumulation.

Moreover, wild-type and D3R-KO cultures, exposed to GD for 1 h, were cultured with nicotine (10 μM) for 48 and 120 h and analyzed for alpha-syn accumulation and morphology.

2.4. Human iPSC-derived DA neurons differentiation and treatments

Human iPSC were obtained from fibroblasts of a healthy donor and characterized as previously described (Bono et al., 2018). Informed consent was obtained from the donor prior to cell donation. The local ethics committee (CEIOC-"San Giovanni di Dio"- Fatebenefratelli Hospital, Brescia, Italy, 44/2001 and 39/2005), previously approved this consent form.

A modified version of the dual-SMAD inhibition protocol was used to obtain mature midbrain DA neurons (Bono et al., 2018; Kriks et al., 2011). hiPSC-derived DA neurons (day 50 of differentiation) were exposed to GD for 1 h and then to nicotine (10 μM) for 48 h, in the presence or in the absence of both TAT-D3R (1 μM) and TAT-β2 (1 μM) peptides. In parallel, treatment with nicotine was performed in the presence of the scrambled peptides TAT-D3R-sc and TAT-β2-sc (both at 1 μM). In some experiments, human DA neuron cultures exposed to GD for 1 h, were treated with nicotine (10 μM) for 48 h, in the presence or in the absence of the PI3K inhibitor LY294002 (10 μM), added 30 min before nicotine treatment. Neurons were fixed and analyzed for alpha-syn accumulation by immunofluorescence.

2.5. Immunofluorescence and confocal microscope analysis

Neurons were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (Sigma-Aldrich), blocked in PBS containing 0.1% Triton X-100 (Promega, Madison, WI, USA) and 5% bovine serum albumin (BSA; Sigma Aldrich), and incubated overnight at 4 °C with primary antibodies. The following antibodies were used: anti-tyrosine hydroxylase (TH, 1:700; Santa Cruz Biotechnology) and anti-alpha-syn 211 (1:500; Santa Cruz Biotechnology). As a negative control, the primary antibodies were omitted. Neurons were then incubated for 30 min at room temperature with appropriate Alexa Fluor 488- and Cy3-conjugated secondary antibodies (Jackson Immuno Research). Nuclei were stained with DAPI. Coverslips were mounted with Vectashield Mounting Medium (Vector Laboratories). For thioflavin-S staining, cells were incubated with 0.05% thioflavin-S in PBS, washed three times for 5 min with 80% ethanol (EtOH), and then blocked for subsequent immunostaining. Images were captured using a Zeiss LSM 880 confocal microscope equipped with Plan-Apochromat 63×/1.4 numerical aperture oil objective and examined using Zen 2.3 Software (Carl Zeiss AG, Oberkochen, Germany). Alpha-syn quantification was performed as previously described (Bono et al., 2018). A minimum of 10 fields containing TH-positive neurons for each condition were selected and analyzed by using the NIH IMAGE J Software (NIH, Bethesda, MD, USA); the threshold set-up was fixed between 30 and 200. The densitometric analysis is indicative of the percent average size of the alpha-*syn*-positive areas in the different fields analyzed. For image analysis, each experimental condition was performed in triplicate at least three times.

2.6. Immunocytochemistry and computer-assisted morphology

Neurons were fixed in methanol for 10 min at 4 °C, blocked in PBS containing 0.1%Triton x-100 (Sigma Aldrich) and 5% BSA and incubated o/n at 4 °C with anti-TH antibody (1:500). Neurons were then incubated with the biotinylated anti-rabbit antibody (1:700; 30 min at room temperature) followed by incubation with avidin-biotin horseradish peroxidase complex (Vector). Staining with peroxidase was performed by incubation in PBS containing 1% 3–3′ diaminobenzidine and 0.01% H_2O_2 (Sigma-Aldrich). Digital images were acquired with an Olympus IX51 microscope connected to an Olympus digital camera. Morphometric measurements were performed by a blinded examiner on digitalized images using NIH IMAGE J Software (NIH, Bethesda, MD, USA). The maximal dendrite length, the number of primary dendrites and the soma area were used as indicators of morphological parameters as previously described (Bono et al., 2018). Three slides per treatment group were examined to obtain measurements from at least 30 neurons.

2.7. Proximity Ligation Assay (PLA)

In situ PLA was performed by using the Duolink in situ detection kit (Sigma-Aldrich), according to the manufacture's instructions, as previously reported (Bontempi et al., 2017). PLA was carried out in hiPSC-derived neurons either in the absence or in the presence of both TAT-D3R (1 μM) and TAT-β2 (1 μM), added 24 h before performing PLA. In parallel, PLA was performed on hiPSC-derived neurons incubated for 24 h with the scrambled peptides TAT-D3R-sc and TAT-β2-sc (both at 1 μM). Briefly, cultured human neurons (day 50 of differentiation) were fixed in PBS containing 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature, washed in PBS and blocked with blocking solution (Duolink, Sigma-Aldrich) for 30 min at 37 °C. Neurons were next incubated o/n at 4 °C with goat polyclonal anti-D3R (1:50 dilution; Santa Cruz Biotechnologies) and rabbit monoclonal anti-α4 nAChR subunit (1:100 dilution; Sigma Aldrich) antibodies. According to our previous study (Bontempi et al., 2017), PLA was performed by using the primary antibody against the α4 subunit, since commercial antibodies recognizing the β2 subunit are not recommended for immunofluorescence. After washing, neurons were incubated with anti-rat MINUS and anti-goat PLUS probes (Duolink, Sigma Aldrich) for 30 min at 37 °C, followed by ligation and amplification reaction using Duolink far red detection reagent. After washing, neurons were incubated with the anti-TH antibody (1:500 dilution; Santa Cruz Biotechnologies) for 2 h followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:750 dilution; Jackson Laboratories) for 30 min. Cells were then mounted using mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Duolink, Sigma-Aldrich) and analyzed using a Zeiss LSM 880 confocal microscope equipped with Plan-Apochromat 63×/1.4 numerical aperture oil objective and examined using Zen 2.3 Software (Carl Zeiss AG, Oberkochen, Germany).

2.8. Proteasome activity assay

Proteasome activity was measured using a commercially available Proteasome Activity Assay Kit (Abcam, Cambridge, UK) following the manufacturer's instructions. Briefly, mouse primary neurons were exposed to GD for 1 h and then treated with quinpirole (10 μM) or nicotine (10 μM) or nicotine in the presence of LY294002 (10 μM) for 48 h. Neurons were then lysed in 0.5% NP-40 and incubated with the proteasome substrate for 30 min at 37 °C. Outputs were measured in a fluorometric microplate reader (EnSight Multimode Plate Reader, Perkin Elmer) at Ex/Em 350/440 nm. The values were normalized to maximum inhibition values achieved under the same conditions by adding the MG-132 proteasome inhibitor.

2.9. Statistical analysis

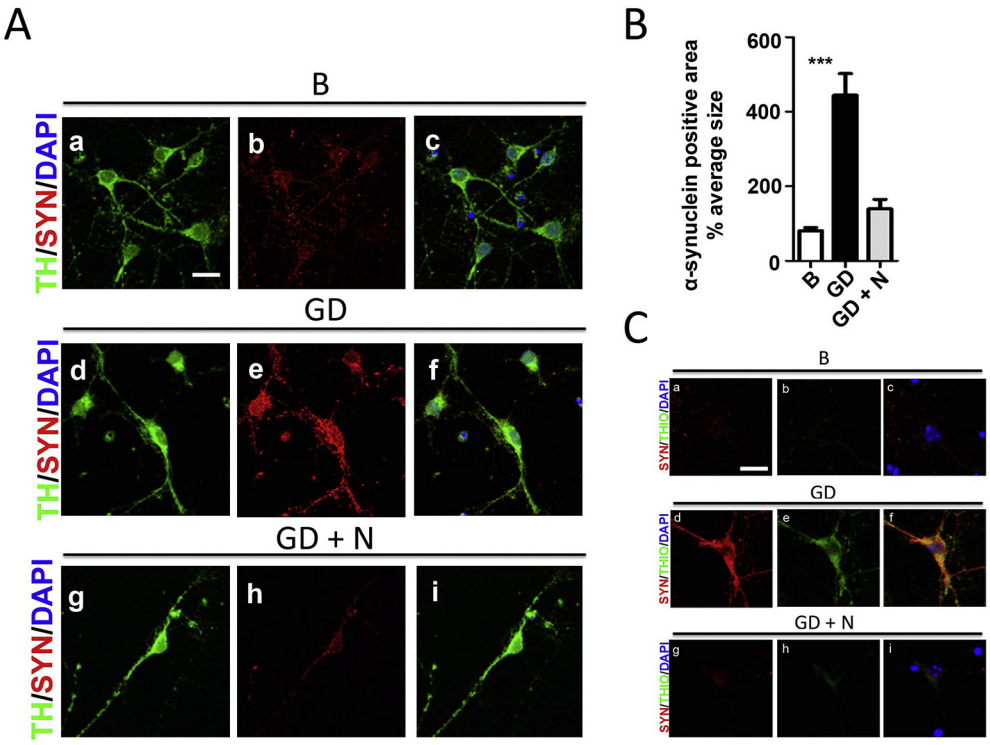
Each experiment was repeated at least three times. Values are expressed as mean ± standard error of the mean (S.E.M.). If not stated otherwise, significant differences from control conditions were determined using analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons provided by GraphPad prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Correlations were assessed by calculating the correlation coefficient between two variables using the same statistical package.

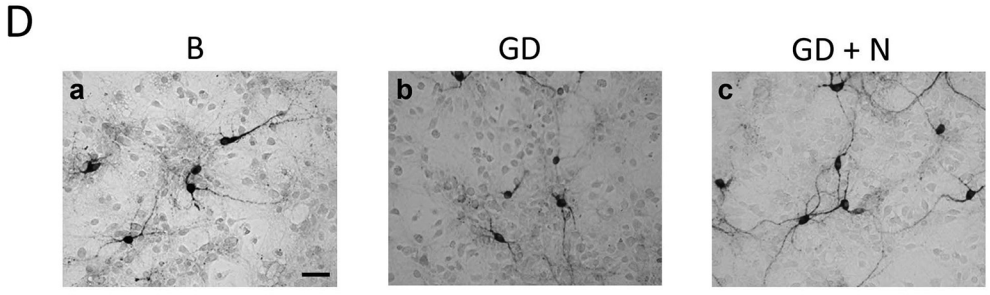
3. Results

3.1. Nicotine prevents both alpha-syn accumulation and morphological defects induced by glucose deprivation (GD) in mouse DA neurons

The ability of nicotine in preventing alpha-syn accumulation was investigated by using primary cultures of mouse mesencephalic neurons, containing DA neurons that endogenously express the D3R-nAChR heteromer (Bontempi et al., 2017).

Neuronal cultures were exposed to GD for 1 h and then cultured in the absence or in the presence of nicotine (10 μM) for 48 h. DA neurons, identified by TH staining, were analyzed for alpha-syn accumulation by immunofluorescence (Fig. 1A and B). As shown in Fig. 1A (panels a–c), untreated TH-positive DA neurons showed a broad distribution of alpha-syn, both at cell body and dendrite levels. GD significantly modified alpha-syn immunolabeling, leading to the formation of numerous dot-like inclusions suggestive of alpha-syn aggregation (Fig. 1A, panel d–f). Remarkably, nicotine treatment significantly prevented GD-induced alpha-syn accumulation in TH-positive neurons (Fig. 1A, panel g–i). To assess whether alpha-syn aggregates had a fibrillary structure, reminiscent of the LBs pathological inclusions (Morris and Geller, 1996), the fluorescent marker thioflavin S was used (Rideout and Stefanis, 2002). The results show that alpha-*syn*-positive inclusions, observed in GD-exposed neurons, but not in untreated or in GD-exposed and nicotine-treated neurons, were also positive for thioflavine S staining (Fig. 1C). Neurons were also exposed to GD for 1 h, and then to nicotine (10 μm) for 48 and 120 h and analyzed for morphometric parameters, as the average maximal length of the primary dendrite, the dendrite number and the soma area (Bontempi et al., 2017; Collo et al.,





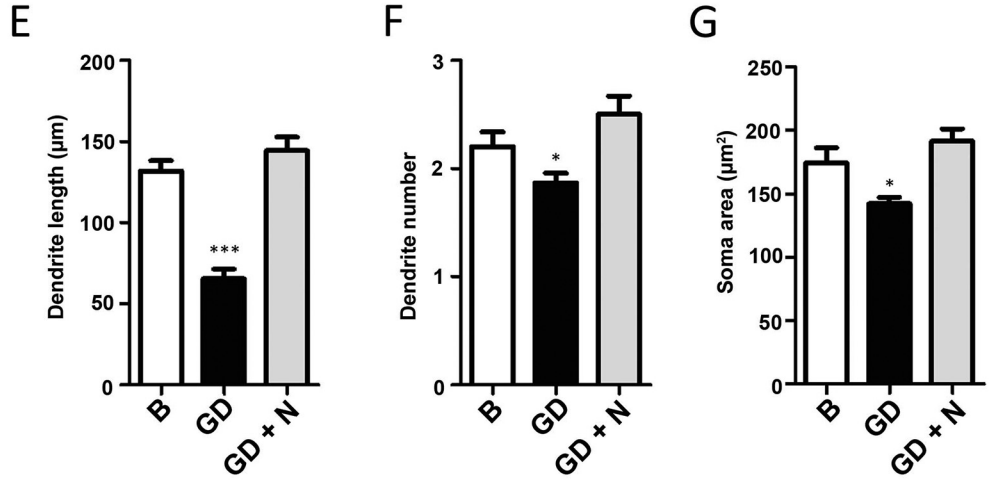


Fig. 1. Nicotine prevents glucose deprivation (GD)-induced alpha-syn accumulation in mouse DA neurons (A) Mesencephalic DA neurons were exposed to glucose deprivation (GD) for 1 h and then were incubated with nicotine (N; 10 μM) for 48 h. Tyrosine hydroxylase (TH, green) and alpha-syn (SYN; red) were analyzed by immunocytochemistry and confocal analysis. Nuclei were stained with DAPI. Scale bar: 20 μm (B). Quantification of the percentage average size of the alpha-syn-immunopositive area in the different conditions. Bars represent mean ± S.E.M. $^{***}p < .001$ vs basal (B). Data were analyzed by one-way ANOVA followed by *post-hoc* comparison with Bonferroni test. (C) Alpha-syn-immunopositive inclusions (red) were co-stained with thioflavin-s (green) and analyzed by immunocytochemistry and confocal microscopy. Nuclei were stained with DAPI. Scale bar: 10 μm (D) Mesencephalic DA neurons were exposed to GD for 1 h and then to nicotine (N; 10 μM) for 120 h. Representative microphotographs of TH-positive neurons in basal conditions (B), GD, and GD followed by 120 h exposure to nicotine (N; 10 μM). Scale bar: 60 μm. (E-G) Morphologic effects induced by GD and GD followed by nicotine on maximal dendrite length (E), number of primary dendrites (F), and soma area (G). Bars represent the mean ± S.E.M. $^{***}p < .001$, $^{*}p < .05$ vs basal. Data were analyzed by one-way ANOVA followed by *post-hoc* comparison with Bonferroni test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2008) (Fig. 1D–G). Analysis of TH-positive neurons 48 post GD did not show significant morphological alterations (data not shown). By contrast, a significant reduction of the length of the primary dendrite and of both dendrite number and soma area was observed 120 h post GD (Fig. 1E–G). Thus, the first event induced by GD in DA neurons is alpha-syn accumulation (48 h) followed by a delayed neuronal injury (120 h). Interestingly, also the structural defects observed 120 h post GD were significantly prevented by nicotine (Fig. 1E–G).

3.2. Nicotine-mediated inhibition of alpha-syn accumulation induced by GD in mouse DA neurons requires the D3R-nAChR heteromer and D3R signaling

The role of D3R-nAChR heteromer in counteracting nicotine-induced alpha-syn accumulation was next investigated. First, midbrain

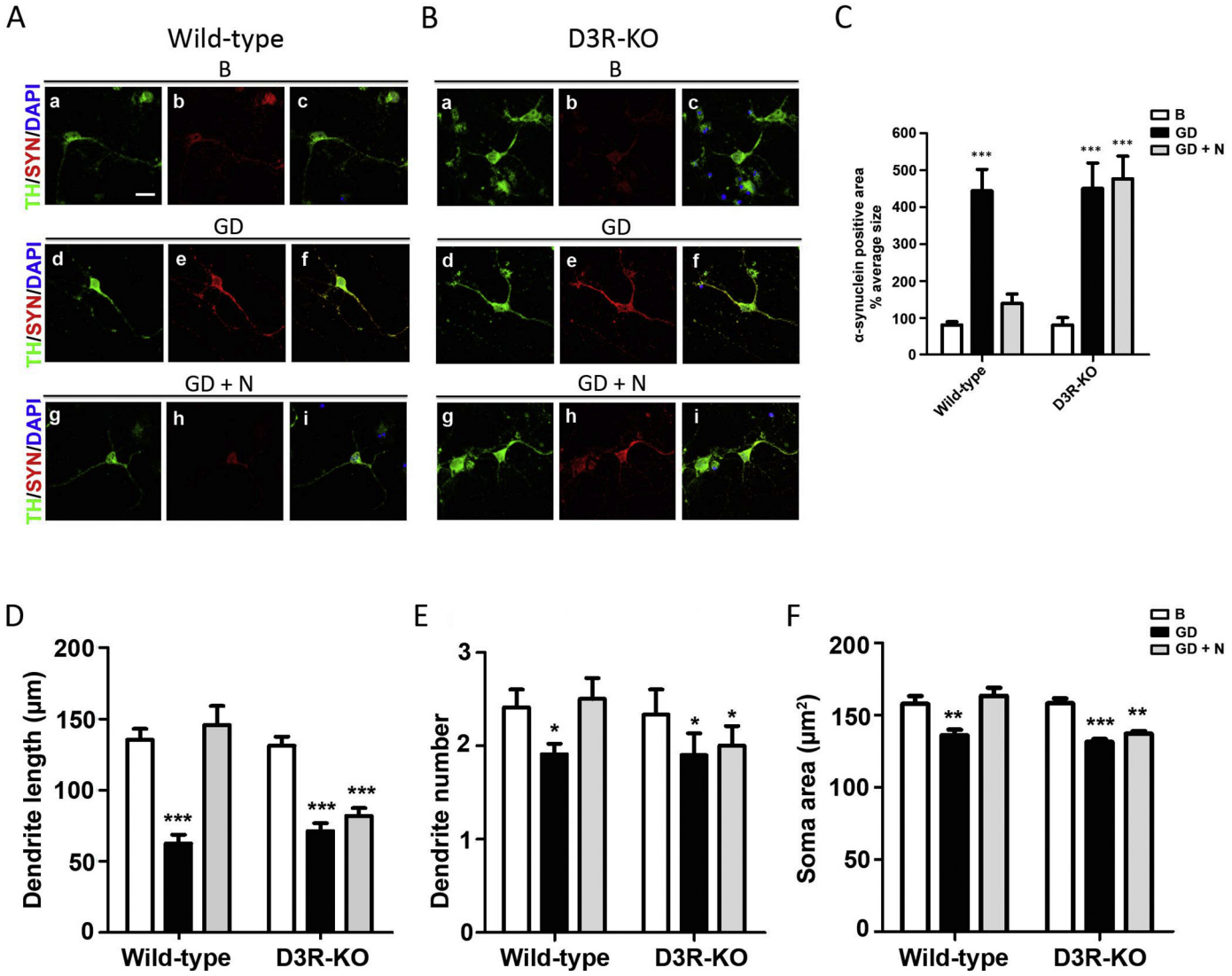


Fig. 2. Nicotine inhibition of GD-induced alpha-syn accumulation in mouse DA neurons requires the D3R. (A–B) Mesencephalic DA neurons from wild-type (A) and D3R-KO (B) mice were exposed to GD for 1 h followed by incubation with nicotine (N, 10 μM) for 48 h. Analyses of TH (green) and alpha-syn (SYN; red) were performed by immunocytochemistry and confocal analysis. Nuclei were stained with DAPI. Scale bar: 20 μm. (C) Quantification of the percentage average size of the alpha-*syn*-immunopositive area in the different conditions. Bars represent mean ± S.E.M. $^{***}p < .001$ vs basal (B). Data were statistically analyzed by two-way ANOVA followed by *post-hoc* comparison with Bonferroni test. (D-F) Mesencephalic DA neurons from wild-type and D3R-KO mice were exposed to GD for 1 h followed by incubation with nicotine (N, 10 μM) for 120 h. TH-positive neurons were analyzed for maximal dendrite length (D), number of primary dendrites (E), and soma area (F). Bars represent the mean ± S.E.M. $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$ vs basal. Data were analyzed by one-way ANOVA followed by post-hoc comparison with Bonferroni test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neurons obtained from both wild-type and D3R-KO mice (D3R-KO neurons), were exposed to GD for 1 h and then to nicotine (10 μM) for 48 h and analyzed for alpha-syn accumulation (Fig. 2A–C). As reported in Fig. 2A and B, in D3R-KO TH-positive neurons, but not in wild-type ones, GD-induced formation of alpha-syn inclusions was insensitive to the inhibitory effects of nicotine (Fig. 2B, panel d–f and a–c, respectively). In addition, neurons from both wild-type and D3R-KO mice exposed to GD for 1 h, and then to nicotine (10 μm) for 120 h, were analyzed for morphometric parameters (Fig. 2D–F). Analysis of TH-positive neurons revealed that in D3R-KO neurons nicotine was unable to prevent the significant reduction of the length of the primary dendrite and of both dendrite number and soma area induced by GD, thus supporting that the effects of nicotine are dependent on the D3R.

Next, the specific role of D3R-nAChR heteromer in mediating these effects was demonstrated by using two different cell-permeable interfering peptides, previously found to disrupt the D3R-nAChR complex (Bontempi et al., 2017; Matera et al., 2019). In particular, the TAT-D3R and the TAT-β2 cell-permeable interfering peptides and the corresponding scrambled peptides (TAT-D3R-sc and TAT-β2-sc) were used (Bontempi et al., 2017; Matera et al., 2019). Mouse midbrain neurons were exposed to GD (1 h) and then cultured with nicotine (10 μM) for 48 h, in the presence or in the absence of either TAT-D3R (1 μM) or TAT-β2 (1 μM). In parallel, treatment with nicotine was performed in the presence of the scrambled peptides TAT-D3R-sc and TAT-β2-sc (both at 1 μM) (Fig. 3A and B). The results show that the inhibitory effect of nicotine on alpha-syn accumulation induced by GD was lost in the presence of either TAT-D3R or TAT-β2 peptides (Fig. 3A, panel j–l and p–r, respectively), while it was preserved in the presence of TAT-D3R-sc and TAT-β2-sc peptides (Fig. 3A, panel m–o and s–u, respectively). Moreover, GD-exposed neurons (1 h) were treated with nicotine (10 μM) for 120 h in the presence or in the absence of either TAT-D3R (1 μM) or TAT-β2 (1 μM) or their scramble counterparts (TAT-D3R-sc and TAT-β2-sc, both at 1 μM) and the TH-positive neurons were analyzed for morphometric parameters (Fig. 3C–E). We found that the

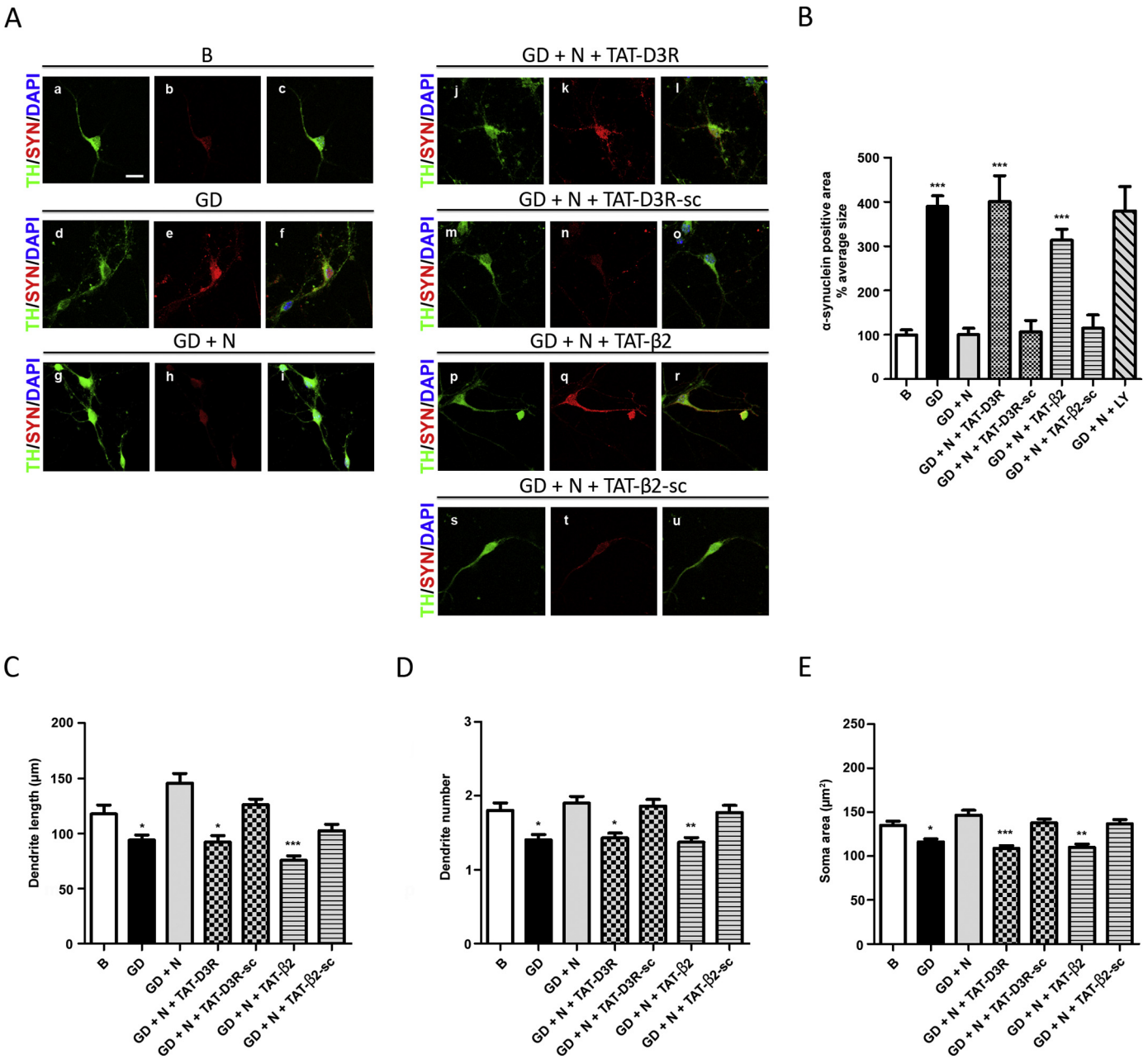
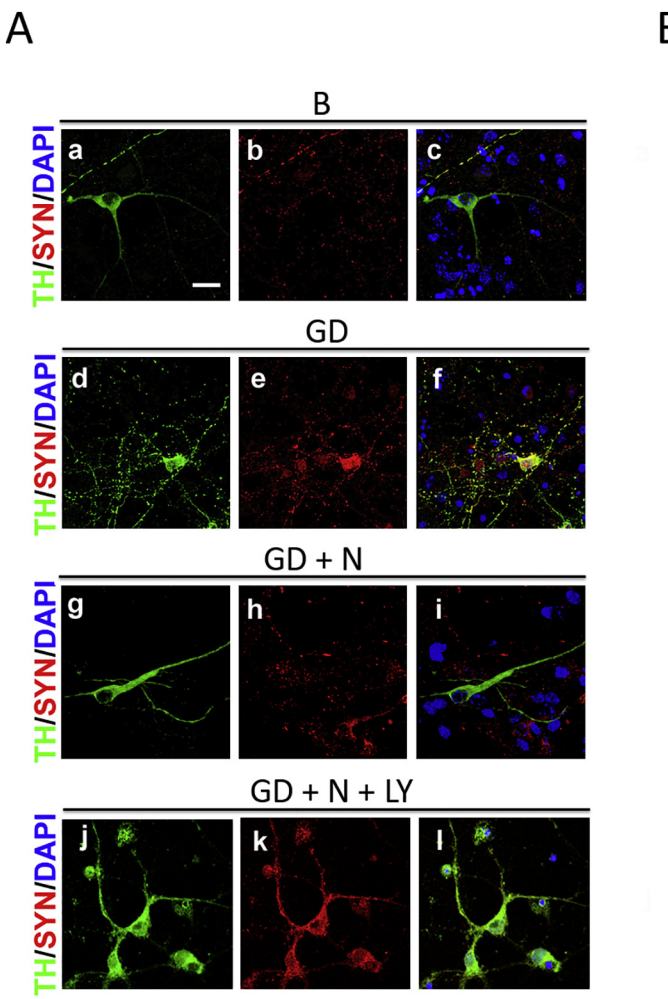


Fig. 3. Nicotine inhibition of GD-induced alpha-syn accumulation and morphological defects in mouse DA neurons requires the D3R-nAChR heteromer. (A) DA neurons were exposed to GD for 1 h (GD) followed by incubation with nicotine (N; 10 μM) for 48 h in the presence or in the absence of TAT-D3R (1 μM), TAT-β2 (1 μM), TAT-D3R-sc (1 μM), or TAT-β2-sc (1 μM) peptides, added 30 min prior to nicotine. TH (green) and alpha-syn (SYN; red) were evaluated by immunocytochemistry and confocal analysis. Nuclei were stained with DAPI. Scale bar: 20 μm. (B) Quantification of the percentage average size of the alpha-*syn*-immunopositive area in the different conditions. Bars represent mean ± S.E.M. $^{***}p < .001$ vs basal (B). Data were analyzed by one-way ANOVA followed by *post-hoc* comparison with Bonferroni test. (C-E) Mesencephalic DA neurons were exposed to GD for 1 h followed by incubation with nicotine (N; 10 μM) in the presence or in the absence of TAT-D3R (1 μM), TAT-β2 (1 μM), TAT-D3R-sc (1 μM), or TAT-β2-sc (1 μM) peptides for 120 h. Mesencephalic DA neurons were analyzed for maximal dendrite length (C), number of primary dendrites (D), and soma area (E). Bars represent the mean ± S.E.M. $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$ vs basal. Data were analyzed by one-way ANOVA followed by *post-hoc* comparison with Bonferroni test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ability of nicotine in counteracting GD-induced morphological alterations was lost in the presence of both TAT-D3R and TAT-β2 peptides but not in the presence of TAT-D3R-sc and TAT-β2-sc, suggesting that in mouse DA neurons the D3R-nAChR heteromer is crucially required for nicotine to prevent both accumulation of alpha-syn and the structural defects induced by GD.

The intracellular signaling pathways involved in nicotine-induced inhibition of alpha-syn accumulation were also investigated. In particular, the possible role of phosphoinositide 3-kinase- (PI3K) dependent signaling was especially analyzed given its association with the D3R (Collo et al., 2008; Collo et al., 2013). Interestingly, this signaling pathway has been also linked to nicotine-mediated structural remodeling of DA neurons (Bono et al., 2018; Collo et al., 2013). Primary cultures of mouse midbrain neurons were exposed to GD for 1 h and then to nicotine (10 μM) for 48 h, either in the absence or in the presence of the PI3K inhibitor, LY290042 (10 μM). Analysis of alpha-syn accumulation was performed in TH-positive neurons (Fig. 4A–B). As previously shown, GD significantly induced alpha-syn accumulation



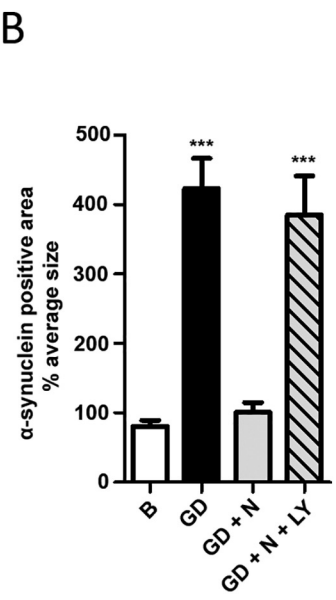


Fig. 4. Nicotine inhibition of GD-induced alpha-syn accumulation in mouse DA neurons requires PI3K signaling. Mesencephalic DA neurons, exposed to GD for 1 h, were incubated with nicotine (N; 10 μM) for 48 h, in the presence or in the absence of the PI3K inhibitor LY 294002 (10 μM), added 30 min prior to nicotine. (A) TH (green) and alpha-syn (SYN; red) were analyzed by immunocytochemistry and confocal analysis. Nuclei were stained with DAPI. Scale bar: 20 μm. (B) Quantification of the percentage average size of the alpha-*syn*-immunopositive area in the different conditions. Bars represent mean ± S.E.M. $^{***}p < .001$ vs basal (B). Data were statistically analyzed by one-way ANOVA followed by *post-hoc* comparison with Bonferroni test.

(Fig. 4A, panel d–f), an effect counteracted by nicotine treatment (Fig. 4A, panel g–i). As reported in Fig. 4A (panels j–l), nicotine-mediated inhibition of alpha-syn accumulation was abolished by blocking the PI3K pathway. Together, these data suggest that nicotine-induced inhibition of alpha-syn accumulation and neuroprotection in mouse DA neurons, requires the D3R-nAChR complex and the activation of PI3K-dependent signaling.

3.3. Nicotine counteracts the impairment of UPS induced by GD through D3R and PI3K signaling

Dysfunction of protein degradation systems, such as the ubiquitine-proteaseome system (UPS), and the resulting accumulation of misfolded proteins has been implicated in the pathogenesis of both familiar and sporadic forms of PD (Olanow and McNaught, 2006).

On this basis, we investigated whether GD and the resulting accumulation of misfolded alpha-syn may affect UPS in mouse midbrain neurons. Neuronal cultures were exposed to GD for 1 h and then to either quinpirole (10 μM) or nicotine (10 μM) for the next 48 h. UPS activity was measured by using a commercial Proteasome Activity Assay kit. As shown in Fig. 5, UPS activity was significantly impaired in GD-treated neurons compared to control (% of UPS activity vs basal: 81 ± 2.2; $p < .01$). This effect was reverted by both quinpirole (% of UPS activity vs basal: 103 ± 1.9) and nicotine (% of UPS activity vs basal: 116 ± 6.1). Interestingly, the ability of nicotine to restore UPS activity was prevented by LY290042 (% of UPS activity vs basal: 82 ± 4.6; $p < .05$) (Fig. 5), suggesting the involvement of D3R-mediated PI3K signaling.

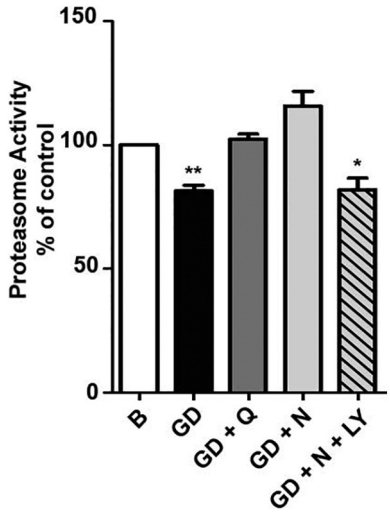


Fig. 5. In mouse neuronal cultures nicotine prevents GD-induced impairment of ubiquitin proteasome system (UPS) through a PI3K-dependent pathway. Mesencephalic DA neurons, exposed to GD for 1 h, were incubated for 48 h with quinpirole (Q; 10 μM) or nicotine (N; 10 μM) or nicotine in the presence the PI3K inhibitor LY 294002 (10 μM), added 30 min prior to nicotine (N; 10 μM). Proteasome activity was determined by using a proteasome activity assay. Bars represent mean ± S.E.M. ($n = 3$) $^{**}p < .01$, $^{*}p < .05$ vs basal (B). Data were statistically analyzed by one-way ANOVA followed by *post-hoc* comparison with Bonferroni test.

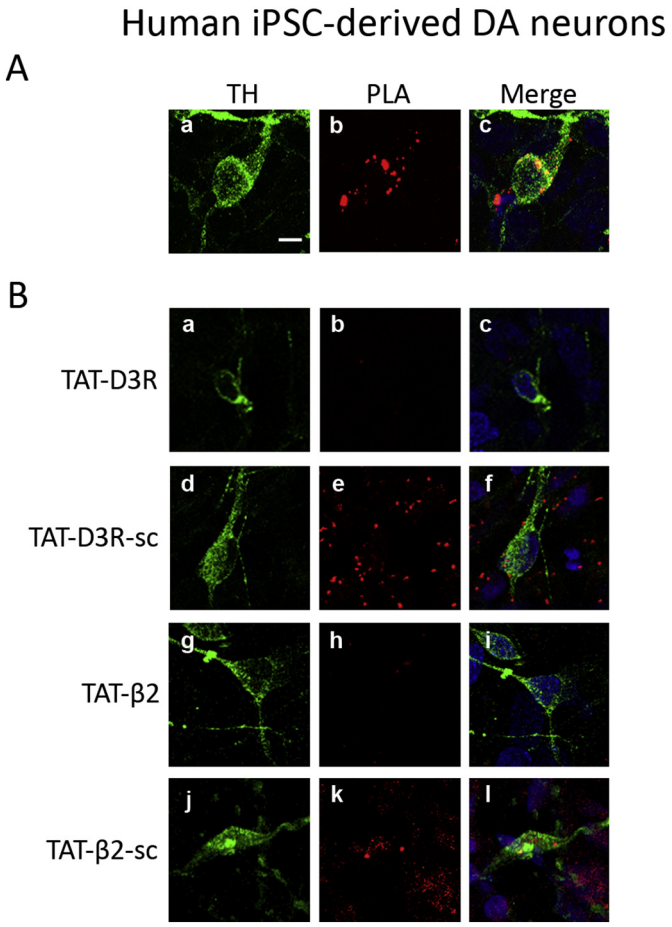


Fig. 6. Detection of D3R-nAChR heteromer by PLA in hiPSC-derived DA neurons. PLA was performed with anti-D3R and anti-α4 nAChR subunit antibodies. (A) Positive PLA signals appear as red spots on TH-positive hiPSC-derived DA neurons (green). Nuclei are detected with DAPI. Scale bar = 20 μm (B) hiPSC-derived cultures were incubated for 24 h with TAT-D3R (1 μM) (panel a–c), TAT-D3R-sc (1 μM) (panel d–f), TAT-β2 (1 μM) (panel g–i) or TAT-β2-sc (1 μM) (panel j–l) followed by PLA analysis. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. The D3R-nAChR heteromer is present in hiPSC-derived DA neurons and mediates the neuroprotective effects of nicotine through D3R-related PI3K pathway

Authentic terminally-differentiated midbrain DA neurons, co-expressing functional D3R and nAChR, were obtained from hiPSC following 50 days of culture (Bono et al., 2018). In this cell model, a cross-talk between nAChR and D3R in counteracting alpha-syn accumulation induced by GD was previously described (Bono et al., 2018). By using "in situ" proximity ligation assay (PLA) (Bellucci et al., 2014; Bontempi et al., 2017), we now investigated whether in human DA neurons, a physical association between the two receptors could occur. PLA was carried out in hiPSC-derived DA neurons at 50 day of differentiation by using primary antibodies for D3R and the α4 subunit of the nAChR (Bontempi et al., 2017) (Fig. 6A and B). As previously described, a primary antibody against the α4 subunit was used in PLA experiments since commercial antibodies recognizing the β2 subunit are not recommended for immunofluorescence. Our BRET results suggested in fact that, even if the α4 subunit does not directly interact with the D3R, its close proximity to the D3R, due to the β2-D3R interaction, is such that a PLA signal can be generated (Bontempi et al., 2017). Confocal images showed that the PLA signal, with a dot-like staining, was mostly located into the soma membranes of TH-positive neurons (Fig. 6A), likely reflecting that in these neuronal domains D3R and nAChR are in close proximity. Moreover, as shown in Fig. 6B, the PLA signal observed in TH-positive neurons was lost when cultures were exposed to either TAT-D3R (panels a–c) or TAT-β2 (panels g–i) interfering peptides (both at 1 μM), but was preserved in the presence of their scrambled counterparts (both at 1 μM) (panels d–f and j–l), suggesting that human DA neurons likely express nAChR-D3R heteromers.

To investigate the mechanism involved in nicotine-induced neuroprotective effect in human neurons, hiPSC-derived DA cultures were exposed to GD for 1 h and then to nicotine (10 μM), in the absence or in the presence of TAT-D3R or TAT-β2 (both at 1 μM) for 48 h. In addition, neuronal cultures exposed to GD, were cultured with nicotine (10 μM; 48 h) in the absence or in the presence of the scrambled peptides, TAT-D3R-sc and TAT-β2-sc (both at 1 μM). TH-positive neurons were then analyzed for alpha-syn accumulation (Fig. 7A and B). Alpha-syn accumulation induced by GD, observed in TH-positive neurons (panel d–f), was strongly counteracted by nicotine treatment (panel g–i); moreover, nicotine protection against alpha-syn accumulation induced by GD was strongly prevented by co-incubating nicotine with either TAT-D3R or TAT-β2 peptides (panel j–l and panel p–r, respectively). Conversely, incubation with nicotine in combination with TAT-D3R-sc and TAT-β2-sc peptides did not affect nicotine-induced prevention of alpha-syn accumulation (panel m–o and s–u, respectively). In addition, hiPSC-derived DA neurons, were exposed to GD for 1 h and then to nicotine (10 μM; 48 h), either in the absence or in the presence of the PI3K inhibitor LY290042 (10 μM). The results showed that nicotine in the presence of LY290042 did not counteract the formation of alpha-syn aggregates (panel v–x).

Together, these data indicate that in human DA neurons, as observed in mouse neurons, the interaction of nAChR with the D3R and the activation of the D3R-dependent PI3K intracellular signaling are critical for nicotine to exert neuroprotection.

4. Discussion

We recently reported that in mouse DA neurons the heteromeric complex formed by the D3R and the nAChR mediates the neurotrophic effects of nicotine (Bontempi et al., 2017). We now show that the D3R-nAChR complex is also present in human DA neurons and that nicotine, by activating this complex, rescues DA neurons from alpha-syn pathological accumulation and neuronal shrinkage.

The GD model of neuronal injury was used to induce cytoplasmic aggregates of alpha-syn in DA neurons; these aggregates were positive for thioflavine-S, thus suggesting a fibrillary-like structure that is typical of the pathological inclusions observed in PD (Rideout and Stefanis, 2002). Alpha-syn accumulation was observed at 48 h post GD and was followed by structural alterations such as reduction of both the dendrite arborization and soma size, which appeared later, at 120 h from GD. By using this model, we found that chronic nicotine administration prevented both the formation of alpha-syn inclusions and the morphological defects induced by GD, thus indicating that nicotine has a neuroprotective effect on DA neurons.

The potential role of nicotine in neuroprotection has been suggested by molecular, cellular and epidemiological studies (Kawamata and Shimohama, 2011; Quik et al., 2012; Subramaniam et al., 2018). In particular, both in vitro and in vivo studies have shown that nicotine, by activating nAChR expressed on DA neurons, strongly prevented neurotoxicity induced by selective toxic agents, such as rotenone, 6-OHDA and MPTP (Kawamata and Shimohama, 2011). α4β2, α6β2 and α7 nAChR subtypes, especially expressed on DA neurons (Champtiaux et al., 2003), have been implicated in nicotine-mediated neuroprotection through the activation of different intracellular pathways, including JAK2/STAT, (Junyent et al., 2010), MEK/ERK (Arredondo et al., 2006; Dajas-Bailador et al., 2002) and PI3K/AKT (Toulorge et al., 2011; Suzuki et al., 2013). More recently, neuroprotection by nicotine

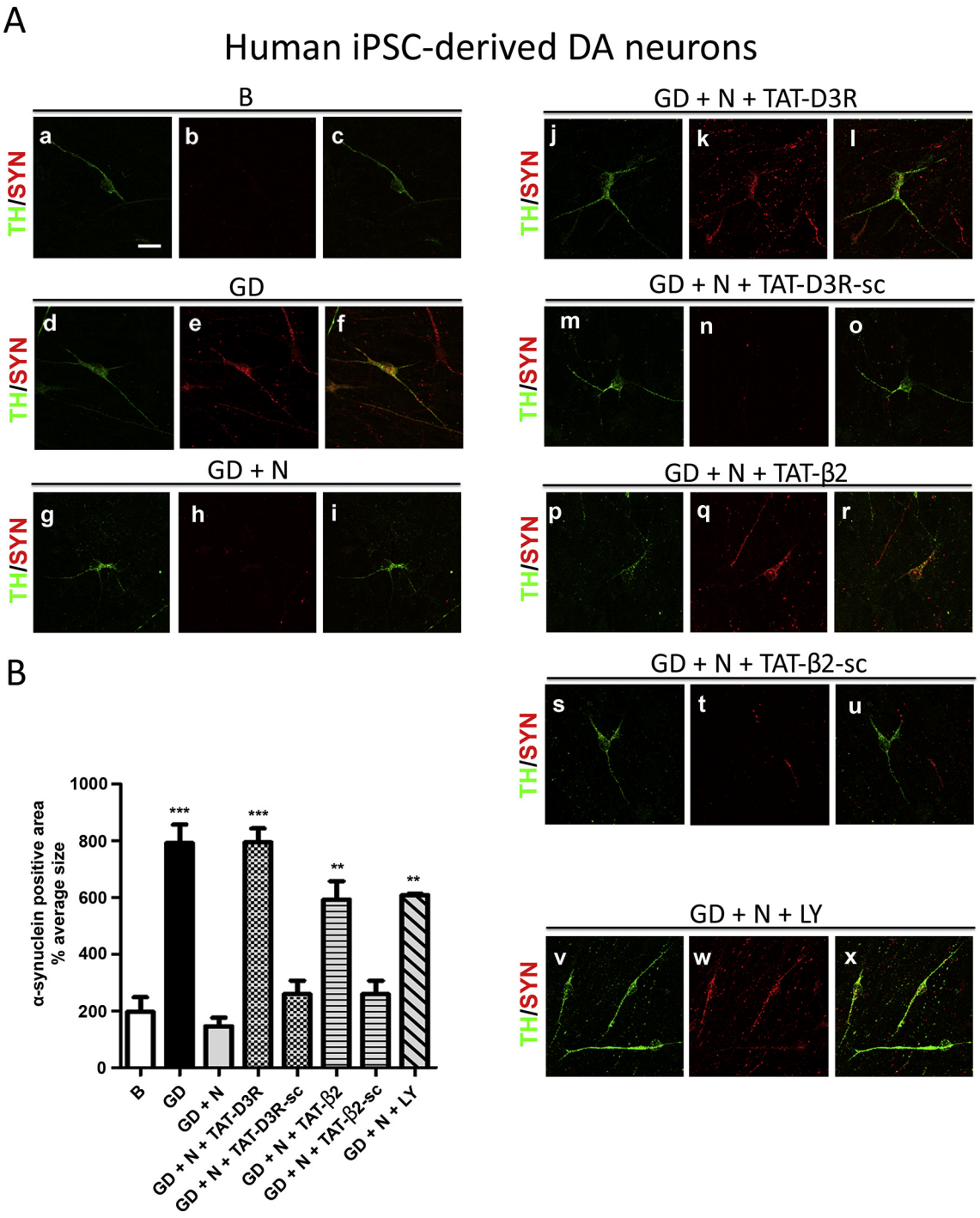


Fig. 7. Nicotine inhibition of GD-induced alpha-syn accumulation in hiPSC-derived DA neurons requires the D3R-nAChR complex. (A) hiPSC-derived DA neurons were exposed to GD for 1 h followed by incubation with nicotine (N; 10 μM) for 48 h, in the absence or in the presence of TAT-D3R (1 μM), TAT-β2 (1 μM), TAT-D3R-sc (1 μM), or TAT-β2-sc (1 μM), added 30 min prior to nicotine. hiPSC-derived DA neurons, exposed to GD for 1 h, were also treated with nicotine (N; 10 μM) for 48 h, in the absence or in the presence of the PI3K inhibitor LY 294002 (10 μM), added 30 min prior treatment with nicotine. TH (green) and alpha-syn (SYN; red) were analyzed by immunocytochemistry and confocal analysis. Scale bar = 20 μm. (B) Quantification of the percentage average size of the alpha-syn immunopositive area in the different conditions. Bars represent mean ± S.E.M. $^{**}p < .01$; $^{***}p < .001$ vs basal (B). Data were statistically analyzed by one-way ANOVA followed by post hoc comparison with Bonferroni test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

through the stimulation of α7 nAChR subtypes has been also associated with the activation of Wnt/beta catenin signals (Liu et al., 2017) and the inhibition of PARP-1 enzyme and caspase-3 cleavage (Lu et al., 2017).

In this study, we now suggest that in DA neurons, the neuroprotective effect of nicotine are mediated by β2-containing nAChR that are physically associated with the D3R in the D3R-nAChR heteromer, that represents the functional receptor unit involved in this effect. Disruption of the D3R-nAChR complex by specific interfering TAT-peptides was, in fact, sufficient to abolish the neuroprotective effects of nicotine. These data are further supported by the observation that in DA neurons derived from D3R-KO mice, nicotine was almost completely ineffective in protecting from alpha-syn accumulation. The role of the D3R-nAChR heteromer in mediating nicotine neuroprotection was

further supported by the observation that accumulation of alpha-syn induced by GD was almost totally prevented by inhibiting the PI3K pathway, a pro-survival signal coupled to GPCR activation (Nakano et al., 2017), including the D3R (Beom et al., 2004; Cussac et al., 1999) and recently implicated in the neurotrophic effects of nicotine (Collo et al., 2013). This observation, together with the data showing that PI3K signaling is associated with α7 nAChR stimulation (Kawamata and Shimohama, 2011; Toulorge et al., 2011) suggests that this pro-survival pathway may be a common intracellular signaling activated by both α7 nAChR and D3R-nAChR complexes. Moreover these data indicate that an intramembrane cooperation between D3R and nAChR within the D3R-nAChR heteromer sustains D3R-induced PI3K signaling and DA neuron protection. On this line, D3R stimulation has been widely associated with neuroprotection (Fiorentini et al., 2015; Joyce and Millan, 2007) through several mechanisms ranging from DA turnover reduction, to antioxidant or anti-apoptotic activities and or neurotrophic factor up-regulation (Albrecht and Buerger, 2009; Cassarino et al., 1998; Li et al., 2010b; Joyce and Millan, 2007; Zou et al., 2000). Recently, the ability to increase the ubiquitin-proteasome activity (UPS) has been suggested as an additional mechanism of neuroprotection induced by D3R (Li et al., 2010a; Li et al., 2010b). UPS, responsible for the degradation of the majority of intracellular proteins, is a crucial pathway for ensuring a healthy cellular environment, minimizing the accumulation of misfolded and nonfunctional proteins (Borissenko and Groll, 2007). Intriguingly, impaired UPS function has been implicated in PD pathogenesis (Myeku and Duff, 2018; Zheng et al., 2014) and a direct inhibition of UPS has been associated with elevated alpha-syn concentration or alpha-syn insoluble aggregates (Chen et al., 2006; Snyder et al., 2003; Zhang et al., 2008). On these bases, we measured UPS activity in mouse neurons and showed that GD-induced alpha-syn accumulation was coincident with a remarkable reduction of UPS activity and that the D2R/D3R agonist quinpirole significantly restored UPS function. Interestingly, we also found that nicotine was equally able to improve GD-induced UPS impairment, an effect specifically prevented by PI3K inhibitors. These data are consistent with previous findings showing that nicotine modulates UPS in different brain areas (Henley et al., 2013; Kane et al., 2004; Massaly et al., 2015; Rezvani et al., 2007). Moreover, the hypothesis that in PD patients nicotine may protect DA neurons by targeting the UPS system has also been suggested (Chapman, 2009). We now suggest that UPS recovery could be a mechanism contributing to nicotine-mediated DA neuron protection that occurs through the activation of nAChR and requires D3R activity. Whether or not this effect is exclusively dependent on nicotine activation of the D3R-nAChR complex remains to be fully elucidated.

The relevance of the D3R-nAChR heteromer in protecting DA neurons form injury has been further supported by the observation that this complex also operates in human neurons. By using the PLA assay, we established that in human DA neurons D3R are in a very close proximity with α4β2-containing nAChR that can be best explained with the formation of the D3R-nAChR heteromer. We also found that nicotine neuroprotective effects were almost completely reverted by interfering TAT-peptides disrupting D3R-nAChR interaction. Moreover, the PI3K inhibitor LY290042 significantly prevented the neuroprotective effect of nicotine on DA neuron underlining the crucial role of D3R signaling and the intramembrane cooperation between D3R and nAChR.

Despite the large amount of evidence suggesting the role of nicotine in protecting DA neurons (Picciotto and Zoli, 2008; Shimohama, 2009), clinical trials and case studies designed for ascertain the potential of nicotine as a disease-modifying drug for PD have generated controversial results (Ma et al., 2017). As shown for nicotine, clinical trials investigating the disease-modifying properties of the D3R-preferring agonist pramipexole in PD patients have failed (Kalia et al., 2015). The late stage of the disease by the time of PD diagnosis and the irreversible damage accumulated by DA neurons could partially explain both D2R/D3R agonists and nicotine treatment failure, consistent with previous studies indicating that nicotine is unable to restore neurons integrity once damage has occurred (Huang et al., 2009). On the other hand, our data showing that UPS activity restoration may contribute to nicotine-induced neuroprotection may support the notion that nicotine could be beneficial only in patients at early stage of PD disease. Impairment of the UPS, in fact, likely represents a triggering event leading to an aberrant and irreversible accumulation of misfolded proteins, such as alpha-syn, that are toxic for DA neurons (Dawson and Dawson, 2003).

5. Conclusions

A large amount of evidence supports the involvement of both α7 and β2-containing nAChR subtypes in nicotine-mediated neuroprotection on DA neurons (Takeuchi et al., 2009; Jeyarasasingam et al., 2002; Toulorge et al., 2011; Yanagida et al., 2008; Suzuki et al., 2013; Bordia et al., 2015); interestingly α7 nAChR have been also detected on non-neuronal cells such as microglia (Suzuki et al., 2013; Stuckenholz et al., 2013) and astrocytes (Liu et al., 2015), suggesting that in addition to a direct role on DA neurons, α7 subtypes may have a special contribution in preventing the DA neuron damage through the modulation of astrocytes and microglia activity.

In this study, we suggest that a receptor mosaic containing the D3R and the β2-nAChR with neuroprotective properties is present in both mouse and human DA neurons. Even if a detailed characterization of the transductional and trafficking properties of the D3R-nAChR complex is still lacking, the relevance of PI3K signaling in D3R-nAChR-mediated neuroprotection is clearly demonstrated; in particular, stimulating the D3R-nAChR complex restores the UPS as a final mechanism contributing to protection against alpha-syn accumulation. The D3R-nAChR complex, by exerting both neurotrophic and neuroprotective effects, is a crucial molecular effector for preserving DA neurons homeostasis, while abnormal activity of the complex could be taken into account when considering the various diseases affecting DA neuronal transmission, including PD or addiction. It is important to highlight that our findings have been validated in a human model of DA neurons that physiologically express the heteromer. Therefore, the D3R-nAChR complex may represent a novel potential target for drugs that, by selectively modulating its activity, might contribute to prevent or slow down DA neuron degeneration, especially in PD patients at the early stages of the disease.

Acknowledgment

We thank Adele Guglielmi for her contribution in hiPSC maintenance and midbrain DA neurons differentiation. Federica Bono was supported by a grant from Fondazione Cariplo, Milano, Italy, Programma Emblematici Maggiori 2014.

Funding

This work was supported by the Italian Institute of Technology Foundation (IIT) (Grant to C.M.) and by grants from the University of Brescia.

Conflict of interest

The authors declare no conflict of interest.

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