Pharmacological Research 160 (2020) 105185

Contents lists available at ScienceDirect

Pharmacological Research

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

Protease-activated receptor 1 (PAR1) inhibits synaptic NMDARs in mouse nigral dopaminergic neurons

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ARTICLE INFO

Keywords:
Protease-activated receptor 1 (PAR1)
Serine proteases
Substantia nigra
Dopamine
NMDARs
Synaptic transmission

ABSTRACT

Protease-activated receptor 1 (PAR1) is a G protein-coupled receptor (GPCR), whose activation requires a proteolytic cleavage in the extracellular domain exposing a tethered ligand, which binds to the same receptor thus stimulating $G\alpha_{q/11}$-, $G\alpha_{i/o}$- and $G\alpha_{12-13}$ proteins. PAR1, activated by serine proteases and matrix metalloproteases, plays multifaceted roles in neuroinflammation and neurodegeneration, in stroke, brain trauma, Alzheimer's diseases, and Parkinson's disease (PD).

Substantia nigra pars compacta (SNpc) is among areas with highest PAR1 expression, but current evidence on its roles herein is restricted to mechanisms controlling dopaminergic (DAergic) neurons survival, with controversial data showing PAR1 either fostering or counteracting degeneration in PD models. Since PAR1 functions on SNpc DAergic neurons activity are unknown, we investigated if PAR1 affects glutamatergic transmission in this neuronal population.

We analyzed PAR1's effects on NMDARs and AMPARs by patch-clamp recordings from DAergic neurons from mouse midbrain slices. Then, we explored subunit composition of PAR1-sensitive NMDARs, with selective antagonists, and mechanisms underlying PAR1-induced NMDARs modulation, by quantifying NMDARs surface expression.

PAR1 activation inhibits synaptic NMDARs in SNpc DAergic neurons, without affecting AMPARs. PAR1-sensitive NMDARs contain GluN2B/GluN2D subunits. Moreover, PAR1-mediated NMDARs hypofunction is reliant on NMDARs internalization, as PAR1 stimulation increases NMDARs intracellular levels and pharmacological limitation of NMDARs endocytosis prevents PAR1-induced NMDARs inhibition.

We reveal that PAR1 regulates glutamatergic transmission in midbrain DAergic cells. This might have implications in brain's DA-dependent functions and in neurological/psychiatric diseases linked to DAergic dysfunctions.

1. Introduction

Protease-activated receptor 1 (PAR1) is a member of a unique family of G protein-coupled receptors (GPCRs), whose activation requires a site-specific proteolytic cleavage in the N-terminal extracellular domain, exposing a tethered ligand which binds to the same receptor [1–4]. Endogenous activators of PAR1 are serine proteases like thrombin, plasmin, activated protein C (APC), factor Xa (FXa), and factor VIIa (FVIIa) [1,3,5]. Additionally, PAR1 can be activated by various matrix metalloproteases (MMPs), including MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13, by proteolysis at non-canonical sites [6–10], transactivated by tethered ligands of homo- or heterodimer partners [11], or activated by synthetic soluble ligands corresponding with cleaved N-terminal sequences, like the peptide TFLLR-NH_2, which represents an invaluable tool to investigate isolated PAR1-mediated actions, without additional side-effects of proteases due to cleavage of other targets.

PAR1 is coupled to different G proteins, $G\alpha_{q/11}$-, $G\alpha_{i/o}$- and $G\alpha_{12-13}$, thus eliciting a complex network of intracellular signaling pathways, including activation of PLC-PKC and MAP kinases, inhibition of AC, and activation of PI3K and Rho kinases [3]. Interestingly, ligand-biased agonism by diverse proteases or synthetic ligands favors a more

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https://doi.org/10.1016/j.phrs.2020.105185
Received 24 April 2020; Received in revised form 22 August 2020; Accepted 26 August 2020
Available online 3 September 2020

segregate activation of different G protein-dependent pathways, possible mediating distinct PAR1-dependent effects [4].

While PAR1 has been classically studied for its role in coagulation, hemostasis, wound healing and inflammation [3,9,12–14], evidence also supports its function in the regulation of brain activity, either in physiological or pathological conditions. Actually, divergent from the previous believe that PAR1 could be activated only in situations, like traumatic brain injuries (TBI) or ischemia, allowing peripheral/circulating serine proteases to enter in the brain by a compromised blood-brain barrier (BBB), now it is recognized that various serine proteases, besides MMPs, able to activate PAR1 are physiologically synthesized in the brain. Notwithstanding, to date investigations have been mainly pointed to reveal PAR1 contribution to foster/worsen neuronal survival following ischemic, traumatic, neuroinflammatory or neurotoxic insults [15–23]. PAR1 function in normal brain has been less examined, but cumulative evidence supports its role in the regulation of synaptic transmission as well as in learning and memory processes and complex behaviors [24–27].

PAR1 is ubiquitously expressed in the brain, either in neurons or in astrocytes and microglia, although with strong differences between areas and cellular populations [4,28–30]. Substantia nigra pars compacta (SNpc) is among brain areas with highest PAR1 expression [31], with PAR1 localization demonstrated in dopamine (DA) neurons from rodent (rat and mouse) and human midbrain [22,31,32]. In SNpc PAR1 appears localized also in astrocytes [22,32], thus suggesting possible additional glial-dependent PAR1-mediated effects in this brain area. In spite of a well-documented expression, the factual role of PAR1 in the regulation of midbrain DAergic neurons is still enigmatic. Current evidence on its roles herein is mainly restricted to mechanisms controlling DAergic neurons survival, with controversial data suggesting that PAR1 either fostering or counteracting neurodegeneration in Parkinson's disease (PD) models [22,32–34]. Evidence of PAR1-mediated effects in SNpc DAergic neurons in physiological conditions is only limited to the observation that PAR1 activation increases ERK phosphorylation in DAergic cells [22].

Since PAR1 roles on the functional activity of SNpc DAergic neurons are still unknown, here we have investigated if PAR1 affects glutamatergic transmission in this neuronal population. Here, we reveal a negative interaction between PAR1 and NMDARs, whereby, PAR1 activation inhibits synaptic NMDARs in nigral DAergic neurons.

2. Materials and methods

2.1. Experimental animals

All procedures were carried out following the guidelines on the ethical use of animals from the Council Directive of the European Communities (2010/63/EU) and were approved by the Animal Care Committee of Santa Lucia Foundation and Italian Ministry of Health (Authorization N° DM81-2014 PR and DM143-2020 PR). Animal studies comply with the ARRIVE guidelines. C57BL6/J mice were bred in our facility and housed in a temperature- (23 ± 1 °C) and humidity controlled environment (45 %–60 % relative humidity), with a 12 h light/dark cycle (lights off at 7 p.m.). Animals were allowed to take food and water ad libitum.

2.2. Midbrain slice preparation

Acute midbrain slices, used to perform electrophysiological and biochemical experiments, were obtained following standard procedures, as described in Ledonne & Mercuri, 2018 [35]. Briefly, male C57BL6/J mice (18–40 days old) were anesthetized with isoflurane and decapitated. The brain was rapidly removed from the skull and a tissue block containing the midbrain was isolated and immersed in cold artificial cerebrospinal fluid (aCSF) at 8−10 °C. The aCSF contained (in mM): NaCl 126, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $NaHCO_3$ 24, glucose 10, saturated with 95 % O_2–5% CO_2 (pH 7.4). Horizontal slices (250 μm thick) of the ventral midbrain were cut using a vibratome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). Slices were maintained in aCSF at 33.0 ± 0.5 °C for 30 min before being transferred in the recording chamber for the electrophysiological recordings or being further processed for the surface expression assay of NMDARs.

2.3. Electrophysiology

Whole-cell patch-clamp recordings of SNpc DA neurons were performed at 33.0 ± 0.5 °C in a recording chamber placed on the stage of an upright microscope (Axioscope FS, Zeiss, Gottingen, Germany), equipped for infrared video microscopy (Hamamatsu, Tokyo, Japan). Slices were continuously perfused at 2.5–3.0 mL/min with aCSF. SNpc neurons, visually selected by their localization and morphology, were identified as DAergic based on the presence of regular spontaneous firing at 1.5–3 Hz (in cell-attached mode). Patch-clamp recordings were performed with glass borosilicate pipettes (6–8 MΩ) (World Precision Instrument, #TW150F-4) pulled with a PP-83 Narishige puller and filled with a solution containing (in mM): Cs-methanesulfonate 115, CsCl 10, $CaCl_2$ 0.45, HEPES 10, EGTA 1, QX-314 5, MgATP 4, NaGTP 0.3 (pH 7.3 with CsOH).

Recordings were made with a Multiclamp 700B amplifier (Molecular Devices, USA) using Clampex software (Molecular Devices, USA) and a the A/D converter Digidata 1440A (Molecular Devices, USA) connected to a computer. Current signals were filtered at 3 kHz and digitized at 10 kHz. A glass pipette monopolar electrode was placed rostral to the DA neurons recorded (100–200 μm). Excitatory postsynaptic currents (EPSCs) were evoked by delivering brief electrical pulses (100–200 μs duration, every 30 s) through a constant current isolated stimulating unit (Digitimer, UK). NMDAR-mediated EPSCs (NMDAR-EPSCs) were recorded at V_H= +40 mV and isolated by using the $GABA_A$ receptor antagonist, picrotoxin (100 μM), the $GABA_B$ receptor antagonist, CGP55845 (1 μM), the D2 receptor antagonist, sulpiride (1 μM), and the AMPAR antagonist, CNQX (10 μM). AMPAR-mediated EPSCs (AMPAR-EPSCs) were recorded at $V_H = -70$ mV, being isolated by using the same cocktail but replacing CNQX with the NMDAR blocker, MK-801 (10 μM). Amplitude and duration of stimulation pulses were set to obtain synaptic currents of 150–300 pA in baseline. A 2 mV hyperpolarizing step was continuously applied before each synaptic current to monitor changes in access resistance (R_a). Recordings were discarded if R_a changed more than >20 % or holding currents modified more than 100 pA during recordings. NMDAR-EPSCs and AMPAR-EPSCs were analyzed by measuring peak amplitude and data were normalized to the mean of first 10 min (baseline) and expressed as percentage (%).

In a set of experiments, brief applications of NMDA (50 μM, 1 min) or AMPA (10 μM, 45 s) were performed to induce the activation of total (synaptic and extrasynaptic) NMDAR or AMPAR in SNpc DA cells ($V_H = -60$ mV). These recordings of NMDA- and AMPA-activated currents (I_{NMDA} and I_{AMPA}) were made by using a pipette filling solution containing (in mM): K-gluconate 135, KCl 10, $CaCl_2$ 0.05, EGTA 0.1, Hepes 10, NaGTP 0.3, MgATP 4, phosphocreatine 10 (pH 7.3 with KOH). I_{NMDA} and I_{AMPA} were analyzed by measuring peak amplitude and data were normalized to the mean of first three responses (expressed as %). Concentration and duration of drug treatments were designed accordingly with published evidence and previous experience [36].

2.4. Triton insoluble fraction (TIF) preparation

For the extraction of Triton-insoluble postsynaptic fraction (TIF), highly enriched in postsynaptic densities proteins [37], tissues were homogenized with a glass-glass potter in ice-cold buffer containing (in mM) 320 sucrose, 1 HEPES, 1 $MgCl_2$, 1 $NaHCO_3$, 0.1 phenylmethylsulphonylfluoride at pH 7.4 in the presence of Complete™ Protease Inhibitor Cocktail Tablets (Roche Diagnostics) and phosSTOP™ Phosphatase Inhibitor (Roche Diagnostics). The sample was spun at 13

000 g for 15 min at 4 °C. The resulting pellet was resuspended in Triton-KCl buffer (0.5 % Triton™ X-100 and 150 mM KCl) and, after 15 min incubation on ice, it was spun further at 100 000 g for 1 h at 4 °C. The pellet (TIF) was resuspended in 20 mM HEPES buffer supplemented with Complete™ Protease Inhibitor Cocktail tablets and stored at −20 °C. TIF samples for immunoblotting analysis were denatured with Laemmli buffer and subsequent heating (10', 98 °C).

2.5. Surface expression assay

Cross-linking experiments by means of the membrane-impermeable reagent bis-(sulfosuccinimidyl)-suberate (BS^3) were performed to evaluate the NMDAR subunit intracellular pool following PAR1 stimulation. Procedures were performed as previously described [38] with minor modifications. Horizontal midbrain slices containing SNpc (250 μM) were subjected to equal pharmacological treatment as for electrophysiological recordings, being incubated for 30 min in standard aCSF (saturated with 95% O_2–5% CO_2 at 33.5 °C) containing picrotoxin (100 μM), CNQX (10 μM), CGP55845 (1 μM) and sulpiride (1 μM) (control condition) or plus the addition of TFLLR-NH_2 (10 μM, 20 min). After pharmacological treatments, free-floating midbrain slices were incubated in a BS^3 solution (1 mg/mL in PBS) or in PBS (negative control) for 30 min at room temperature in a multiwell on a shaking plate. Then, PBS or BS^3 solution were aspirated and slices were washed three times (10 min each) in cold wash-buffer (PMSF 2 mM, Ethanolamine 50 mM, EDTA 1 mM, NaF 50 mM in PBS). Areas corresponding to SNpc were quickly isolated from the slices and stored at −80 °C for further processing. Tissues were then homogenized at 4 °C with a glass-glass potter in an ice-cold buffer containing 0.32 M Sucrose, 0.1 mM phenylmethylulfonyl fluoride (PMSF), 1 mM Hepes, 1 mM $MgCl_2$, 1 mM NaF supplemented with protease (Complete™, Sigma-Aldrich) and phosphatase (PhosSTOP™, Sigma-Aldrich) inhibitor cocktails. Samples were applied to Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and subjected to immunoblotting analysis.

2.6. Western blotting

TIF and total homogenates proteins were separated on SDS-PAGE followed by western blotting analysis. 10−15 μg of proteins were separated on 6% acrylamide/bisacrylamide gel and transferred on nitrocellulose membrane (Bio-Rad). The membranes were then incubated for 1 h at room temperature in blocking solution (I-block, Tris-Buffered saline (TBS) 1X, 20 % Tween-20) on a shaker and then incubated with the specific primary antibody in blocking solution overnight at 4 C°. The following day, after three washes with TBS and tween 20 (TBS + Tween20 0.1 %; TBSt), they were incubated with corresponding Horseradish Peroxidase (HRP)-conjugated secondary antibody in blocking solution for 1 h at room temperature. After washing with TBSt, membranes were developed using electrochemiluminescence (ECL) reagents (Biorad). Finally, membranes were scanned using a Chemidoc (Biorad Universal Hood III) with Image Lab software (Biorad). Bands were quantified by means of computer-assisted imaging (Image Lab, Biorad). Protein levels were expressed as relative optical density (OD) measurements normalized on tubulin. The primary antibodies used are: anti-GluN1 1:1000 (Thermo Scientific, #32-0500), anti-GluN2B 1:1000 (Invitrogen, #6474), anti-GluN2D 1:500 (Millipore, #MAB5578), anti-tubulin 1:10 000 (Sigma-Aldrich, #T9026).

2.7. Statistical analyses

Numerical data in electrophysiological experiments were expressed as percentage of control mean ± SEM. Data were tested for normality. Statistical comparisons were performed with the unpaired student's *t*-test or one-way ANOVA as appropriate, by using the software SigmaPlot (Systat software Inc, San Diego, USA).

Western blotting data were expressed as percentage of control mean ± SEM. Statistical comparisons were performed with the unpaired Student's *t*-test, using the software GraphPad Prism. The minimal level of statistical significance was set at $P < 0.05$.

2.8. Materials

TFLLR-NH_2, sulpiride and halothane were purchased from Sigma-Aldrich (Milano, IT). NMDA, AMPA, CGP55845, ifenprodil and dynasore were from Tocris (Bristol, UK). CNQX and UBP141 were obtained from Abcam (Cambridge, UK). Picrotoxin and MK-801 were purchased from Hello Bio (Bristol, UK), whilst Vorapaxar (SCH530348) and Atopaxar (E 5555 hydrobromide) were from Axon Medchem (Groningen, The Netherlands). Bis-(sulfosuccinimidyl)-suberate (BS^3) was from Termo Fisher Scientific (#21586).

The following antibodies (Ab) were used: anti-GluN1 (Termo-Scientific, #32-0500), anti-GluN2B (Invitrogen, #6474), anti-GluN2D (Millipore #MAB5578), anti-tubulin (Sigma-Aldrich, #T9026).

3. Results

3.1. PAR1 activation reduces NMDAR-mediated transmission in SNpc DA neurons

To investigate the functional role of PAR1 in the modulation of glutamatergic transmission in midbrain DA neurons, we performed patch-clamp recordings from SNpc DA cells from mouse midbrain slices. Firstly, we analyzed PAR1-induced effects on synaptic NMDARs, by evaluating if the synthetic PAR1 agonist TFLLR-NH_2 affects NMDAR-mediated excitatory postsynaptic currents (NMDAR-EPSCs). We found that perfusion with TFLLR-NH_2 (10 μM, 20 min) reduces NMDAR-EPSCs amplitude in SNpc DA neurons (Fig. 1A) to 82.85 ± 5.30 % of baseline (n = 10). Such TFLLR-NH_2-induced reduction of synaptic NMDARs currents is long lasting, and further decrease is observed during drug washout, being NMDAR-EPSCs reduced to 72.04 ± 9.07 % of baseline at 20 after drug perfusion.

To demonstrate that TFLLR-NH_2-induced reduction of NMDAR-EPSCs is reliant on PAR1 stimulation, we pretreated slices with Vorapaxar, an orthosteric PAR1 inhibitor that prevents receptor activation by occupying the ligand-binding pocket. Such pretreatment with Vorapaxar (5 μM for 10 min prior to TFLLR-NH_2 application) was able to counteract the decrease of NMDAR-EPSCs induced by the PAR1 agonist (Fig. 1C,F). Indeed, mean amplitude of NMDAR-EPSCs in Vorapaxar + TFLLR-NH_2-treated slices was 96.76 ± 1.36 % of baseline (n = 7). Vorapaxar *per se* did not affect NMDAR-mediated transmission, not inducing any modification in NMDAR-EPSCs amplitude (Fig. 1B). To corroborate this evidence, of the selective recruitment of PAR1 in the TFLLR-NH_2-induced reduction of NMDAR-EPSCs, we performed other experiments with a second PAR1 antagonist, Atopaxar. Similarly, we found that Atopaxar (5 μM), while not altering basal NMDAR-mediated synaptic transmission (Fig. 1D), was able to limit TFLLR-NH_2-induced NMDAR-EPSCs reduction (Fig. 1E,F).

To verify that PAR1 stimulation affects total NMDARs conductances in SNpc DA neurons we recorded inward currents induced by application of NMDA, which activates both synaptic and extrasynaptic NMDARs. Inward currents induced by brief applications of NMDA (I_{NMDA}) (50 μM, 1 min) in control conditions were subjected to a slight rundown during repetitive activation (Fig. 2A,B). Notwithstanding, PAR1 stimulation fostered the decrease of I_{NMDA}, which appeared significantly diminished during TFLLR-NH_2 administration (10 μM, 20 min), and further reduced during drug washout (Fig. 2A,B). Actually, mean normalized I_{NMDA} was 56.55 ± 6.11 % in the presence of TFLLR-NH_2 (10 μM, 20 min, n = 7) and 88.07 ± 7.14 % in control conditions. This TFLLR-NH_2-induced NMDAR inhibition was counteracted by Vorapaxar (normalized I_{NMDA} was 78.68 ± 7.56 % in Vorapaxar and TFLLR-NH_2 (n = 7)), and by Atopaxar (normalized I_{NMDA} was 96.97 ± 12.83 % in Atopaxar and TFLLR-NH_2 (n = 4)), thus confirming the involvement of a PAR1-

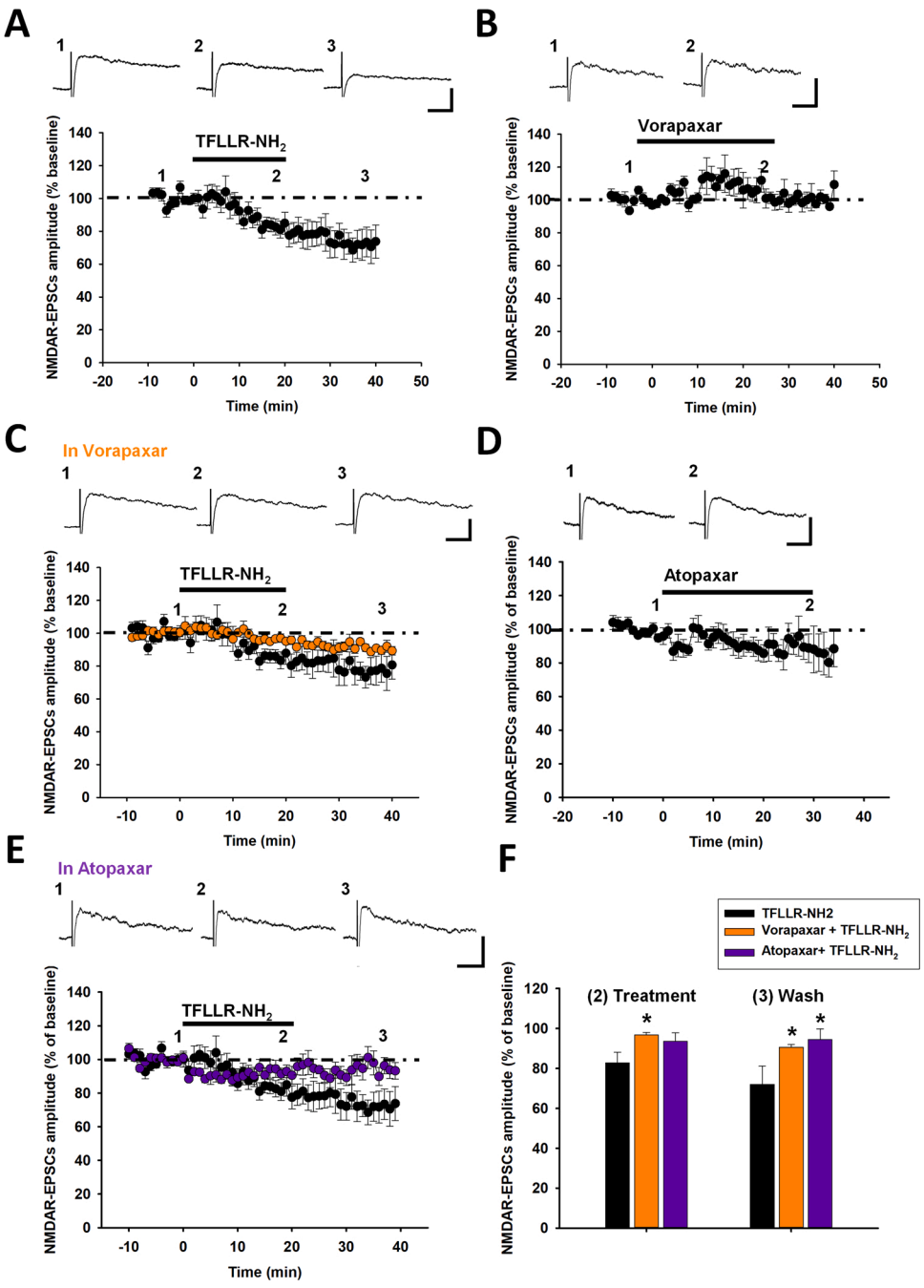

Fig. 1. PAR1 activation inhibits synaptic NMDAR-mediated transmission.
A) PAR1 stimulation, with the PAR1 agonist TFLLR-NH_2 (10 μM, 20 min) reduces NMDAR-mediated excitatory postsynaptic currents (NMDAR-EPSCs) in SNpc DA neurons, as showed in the plot and in the representative traces (upper) of NMDAR-EPSCs: (1) before TFLLR-NH_2, (2) at 20 min of drug perfusion, and (3) 20 min after washout.
B) The orthosteric PAR1 inhibitor, Vorapaxar (5 μM, 30 min), per se does not affect NMDAR-mediated synaptic transmission, whereas **(C,F)** a pretreatment with Vorapaxar (5 μM) for 10 min prior and during TFLLR-NH_2 application (10 μM, 20 min) is able to prevent the decrease of NMDAR-EPSCs induced by the PAR1 agonist.
C) Time course and representative traces of NMDAR-EPSCs (upper) from SNpc DA neurons from midbrain slices treated with Vorapaxar (1), during co-application of TFLLR-NH_2 and Vorapaxar (2), and at 20 min after drug washout (3).
D) Effect of the PAR1 inhibitor Atopaxar (5 μM, 30 min) on basal NMDAR-EPSCs and **(E)** on TFLLR-NH_2-induced reduction of NMDAR-EPSCs.
F) Plot of normalized NMDAR-EPSCs amplitude (as % of baseline) from DAergic neurons in TFLLR-NH_2-, Vorapaxar + TFLLR-NH_2-, and Atopaxar + TFLLR-NH_2-treated slices, showing that preventing PAR1 activation, by Vorapaxar and Atopaxar, significantly antagonizes the TFLLR-NH_2-induced reduction of NMDAR-EPSCs. TFLLR-NH_2 (n = 10 cells/9 mice), Vorapaxar (n = 6 cells/4 mice), Vorapaxar + TFLLR-NH_2 (n = 7 cells/5 mice), Atopaxar (n = 7 cells/5 mice), Atopaxar + TFLLR-NH_2 (n = 10 cells/8 mice). *$p < 0.05$ TFLLR-NH_2 vs Vorapaxar + TFLLR-NH_2, and *p < 0,05, TFLLR-NH_2 vs Atopaxar + TFLLR-NH_2, unpaired t test.
A–E) Scale bar: 100 pA, 50 ms.

dependent mechanism **(**Fig. 2A,B**).**

3.2. AMPAR-mediated transmission is not affected by PAR1 stimulation

We then examined if AMPARs in nigral DA neurons are similarly inhibited by PAR1 activation. We firstly analyzed TFLLR-NH_2's effect on AMPAR-mediated excitatory postsynaptic currents (AMPAR-EPSCs) in these cells. We found that a treatment with TFLLR-NH_2 (10 μM, 20 min) did not modify AMPAR-EPSCs (Fig. 3A**).**

To detect potential PAR1-dependent modulatory effects on extra-synaptic AMPARs, we analyzed inward currents induced by perfusion of AMPA (10 μM, 45 s, each 5 min) in control and during PAR1 stimulation with TFLLR-NH_2. Repeated AMPARs activation did not cause any rundown of AMPA-induced currents (I_{AMPA}), and PAR1 activation did not alter extrasynaptic AMPARs functioning, being I_{AMPA} similarly induced in control and TFLLR-NH_2-treated slices. Mean normalized I_{AMPA} was 102.38 ± 4.3 % during TFLLR-NH_2 application (10 μM, 20 min, n = 6) and 94.37 ± 4.5 in control conditions at the corresponding time point (n = 9) (Fig. 3B**).**

Hence, total AMPARs conductances in SNpc DA neurons, besides synaptic pool, are not affected by PAR1 activation.

3.3. Subunit composition of NMDARs modulated by PAR1

In SNpc DA neurons, functional NMDARs are mainly composed by the association of two GluN1 obligatory subunits with GluN2B and GluN2D subunits [39,40]. To examine if PAR1-dependent inhibition of synaptic NMDARs is conditional to the presence of specific NMDARs subunits, we measured TFLLR-NH_2-induced effects on NMDAR-EPSCs in presence of subunit-specific antagonists, that allow to isolate GluN2B-

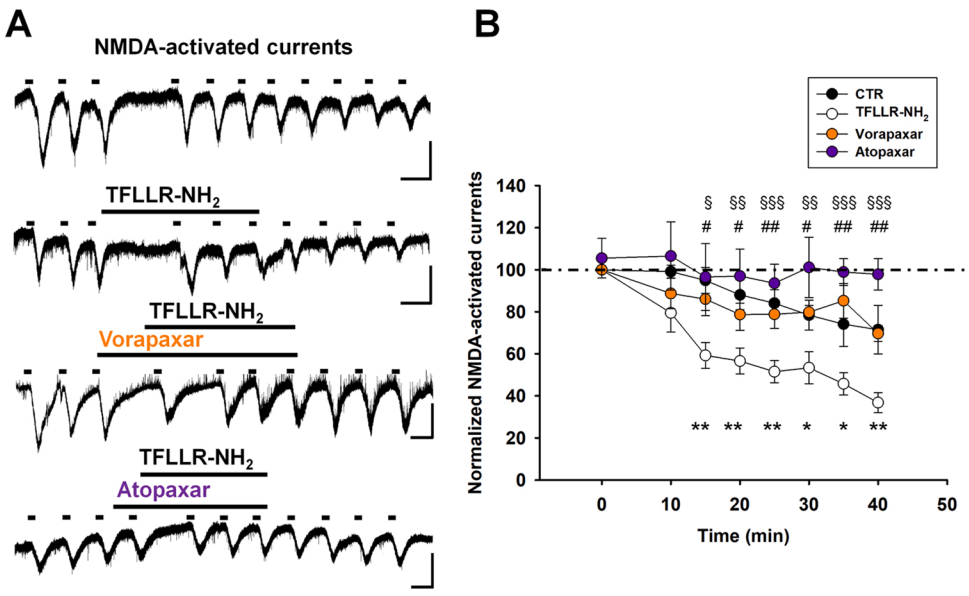

Fig. 2. PAR1 stimulation reduces total NMDARs conductances (synaptic and extrasynaptic).

A) Representative traces of NMDA-activated currents (I_{NMDA}) induced by brief bath applications of NMDA (50 μM, 30 s, every 5 min) in control conditions, or in TFLLR-NH_2-, Vorapaxar + TFLLR-NH_2-, or Atopaxar + TFLLR-NH_2-treated slices. Scale bar: 100 pA, 5 min.

B) Plot of I_{NMDA} peak amplitude (normalized respect the mean of first 3 responses) showing that TFLLR-NH_2 significantly reduces I_{NMDA}, by activating PAR1. The slight run-down of I_{NMDA} in control condition (black dots) is potentiated at each time point by TFLLR-NH_2 (white dots), being this effect prevented by a treatment with either Vorapaxar (orange dots) or Atopaxar (violet dots) prior to TFLLR-NH_2 application. CTR (n = 7 cells/ 7 mice), TFLLR-NH_2- (n = 7 cells/ 5 mice), Vorapaxar + TFLLR-NH_2 (n = 7 cells/5 mice), Atopaxar + TFLLR-NH_2 (n = 4 cells/4 mice).

* $p < 0.05$, ** $p < 0.01$ CTR vs TFLLR-NH_2; # $p < 0.05$, ## $p < 0.01$ TFLLR-NH_2 vs Vorapaxar + TFLLR-NH_2, and §<0.05, §§ $P < 0.01$, §§§ $P < 0.001$ TFLLR-NH_2 vs Atopaxar + TFLLR-NH_2, one-way ANOVA followed by Duncan's test at each time point.

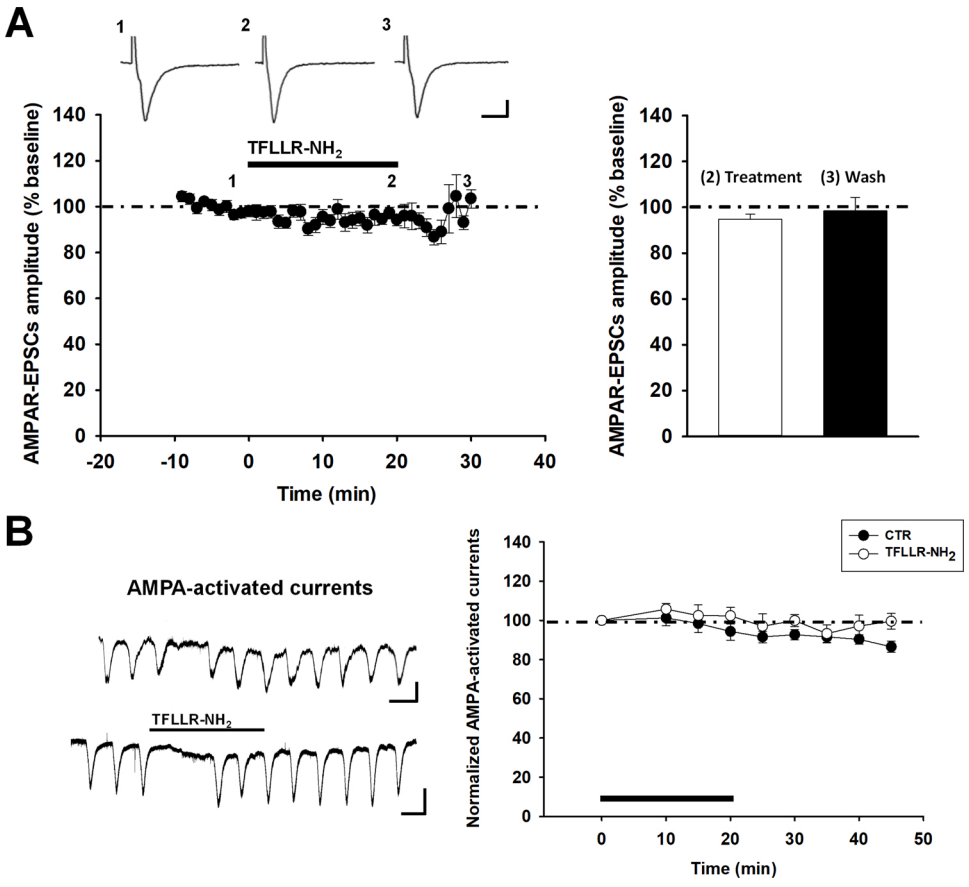

Fig. 3. PAR1 stimulation does not alter AMPAR-mediated transmission.

A) PAR1 activation, with TFLLR-NH_2 (10 μM, 20 min) does not affect synaptic AMPARs, as showed from the representative traces (upper panel) of AMPAR-mediated excitatory postsynaptic currents (AMPAR-EPSCs) from SNpc DA neurons, recorded (1) before TFLLR-NH_2, (2) at 20 min of drug perfusion, and (3) 20 min after washout. (Left panel) Time-course plot of normalized AMPAR-EPSCs peak amplitude and (right panel) histogram of mean AMPAR-EPSCs amplitude during (2) and after (3) TFLLR-NH_2 application. Scale bar: 100 pA, 5 ms.

B) Extrasynaptic AMPARs are not modulated by PAR1 stimulation.

(Left panel) Representative traces of AMPA-activated currents (I_{AMPA}) induced by bath applications of AMPA (10 μM, 45 s, every 5 min) in SNpc DAergic neurons from control and TFLLR-NH_2-treated slices. Scale bar: 100 pA, 5 min.

(Right panel) Plot of I_{AMPA} amplitude (normalized respect to first 3 responses mean) showing that TFLLR-NH_2 (10 μM, 20 min) does not alter I_{AMPA} (white dots), which are similarly induced as in control conditions (black dots). CTR (n = 9 cells/6 mice) and TFLLR-NH_2 (n = 6 cells/5 mice).

and GluN2D-dependent component of NMDAR-EPSCs.

Firstly, we analyzed PAR1 influence on GluN2B-dependent component of NMDAR-EPSCs, isolated by inhibiting specific GluN2D-mediated contribution with the GluN2D antagonist UBP141. In presence of UBP141 (3 μM, 35 min), peak amplitude of NMDAR-EPSCs was reduced to 72.94 ± 5.94 %, representing the isolated GluN2B-mediated component of NMDAR-EPSCs (Fig. 4A). Notably, PAR1 stimulation was able to reduce GluN2B-mediated NMDAR-EPSCs, since TFLLR-NH_2

application in the presence of UBP141 further decreased NMDARs-EPSCS to 53.67 ± 5.79 % (n = 8) (Fig. 4A,B).

Then, we evaluated PAR1-induced effects on GluN2D-dependent component of NMDAR-EPSCs, isolated by inhibiting the GluN2B subunit with the antagonist ifenprodil. Mean NMDAR-EPSCs amplitude in the presence of ifenprodil (3 μM, 35 min) was reduced to 66.11 ± 7.18 %, as expected by inhibiting the GluN2B-dependent component of NMDAR-EPSCs. The consequent PAR1 stimulation with TFLLR-NH_2 induced a reduction of residual NMDAR-EPSCs, indicative of a PAR1-dependent effect on GluN2D-mediated NMDAR-EPSCs component (mean NMDAR-EPSCs amplitude was 52.16 ± 3.35 % of baseline in ifenprodil + TFLLR-NH_2 (n = 11)).

Overall, these results indicate that PAR1 stimulation inhibits GluN2B/GluN2D-containing NMDARs in nigral DAergic neurons.

3.4. PAR1 stimulation does not alter total levels of NMDARs subunits in the postsynaptic density

We then verified if the PAR1-dependent decrease in NMDAR-EPSCs observed in SNpc DAergic neurons is reliant on a reduced NMDARs expression. Thus, we measured expression levels of NMDARs subunits, GluN1, GluN2B and GluN2D, in the postsynaptic compartment in control conditions and following treatments with the PAR1 agonist TFLLR-NH_2. We found that in the Triton-insoluble postsynaptic fraction (TIF) of SN dissected from midbrain slices, a treatment with TFLLR-NH_2 did not alter GluN1, GluN2B, GluN2D levels in the postsynaptic sites (Fig. 5A). This suggest that NMDAR hypofunction in DAergic neurons, caused by PAR1 stimulation, is not dependent by a reduced expression of selective NMDARs subunits in the postsynaptic densities.

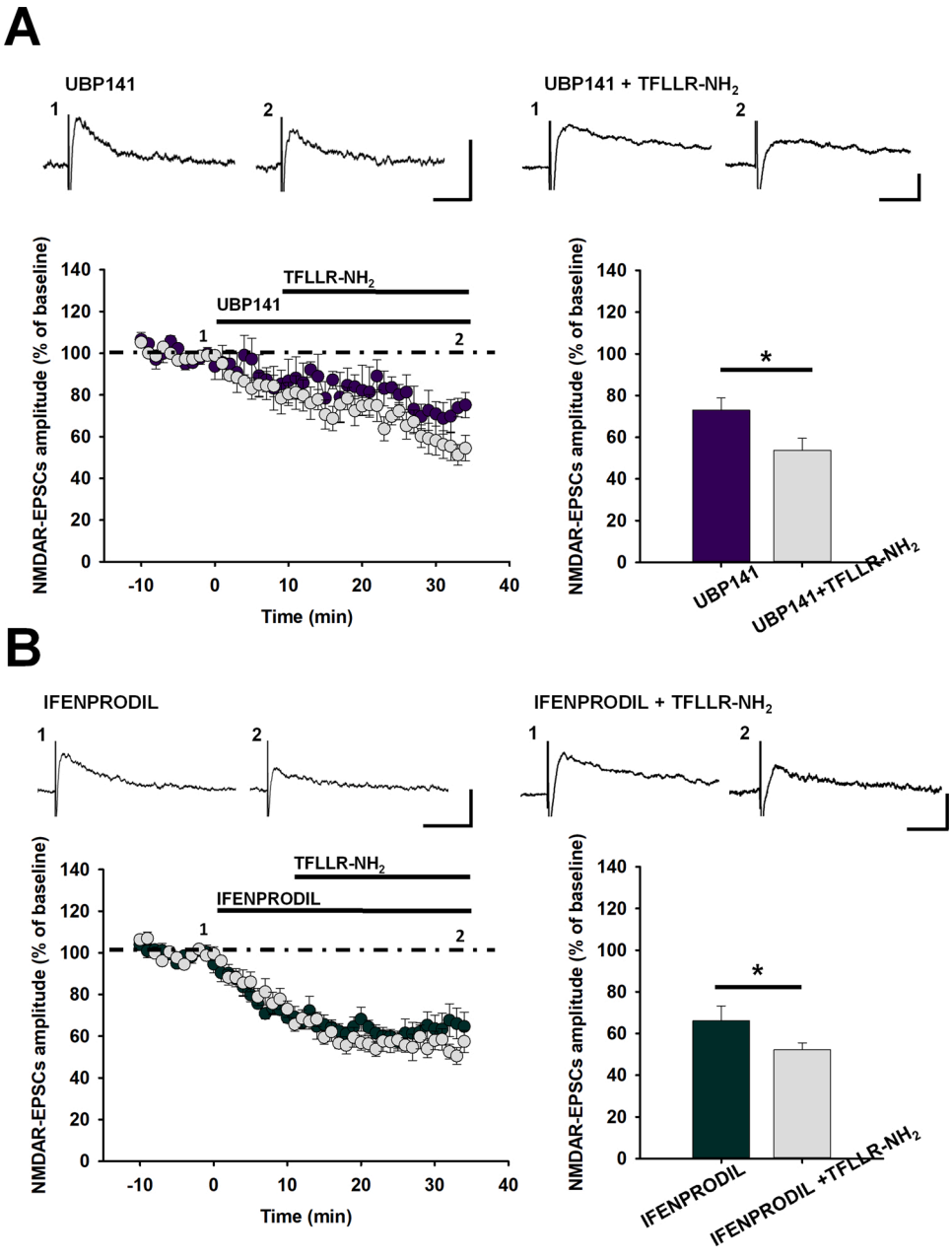

Fig. 4. GluN2B- and GluN2D-containing NMDARs are inhibited by PAR1 activation.
A) Effect of PAR1 stimulation on GluN2B-mediated component of NMDAR-EPSCs.
A treatment with TFLLR-NH_2 reduces GluN2B-mediated NMDAR-EPSCs in SNpc DAergic neurons, as shown in the representative traces (upper panel) of NMDAR-EPSCs recorded (1) before and (2) during treatment with the GluN2D antagonist, UBP141 (3 μM, 35 min) that allows the isolation of a residual GluN2B-mediated EPSCs, or during TFLLR-NH_2 + UBP141 co-application. (Left panel) Time-course plot of normalized NMDAR-EPSCs amplitude and (right panel) histogram of mean normalized NMDAR-EPSCs in the different pharmacological treatments: UBP141 (n = 5 cells/4 mice) and UBP141 + TFLLR-NH_2 (n = 8 cells/5 mice). * $p < 0.05$ UBP141 vs UBP141 + TFLLR-NH_2, unpaired t test. Scale bar: 100 pA, 50 ms.
B) Effect of PAR1 activation on GluN2D-mediated component of NMDAR-EPSCs.
TFLLR-NH_2-induced reduction of GluN2D-mediated NMDAR-EPSCs in SNpc DAergic neurons is shown in the representative traces (upper panel) of NMDAR-EPSCs recorded (1) before and (2) during treatment with the GluN2B antagonist ifenprodil (3 μM, 35 min) that allows the isolation of a residual GluN2D-mediated EPSCs, or during TFLLR-NH_2 + ifenprodil co-application. (Left panel) Time-course plot of normalized NMDAR-EPSCs amplitude and (right panel) histogram of mean normalized NMDAR-EPSCs in the different pharmacological treatments: ifenprodil (n = 8 cells/5 mice) and ifenprodil + TFLLR-NH_2 (n = 11 cells/9 mice). * $p < 0.05$ ifenprodil vs ifenprodil + TFLLR-NH_2, unpaired t test. Scale bar: 100 pA, 50 ms.

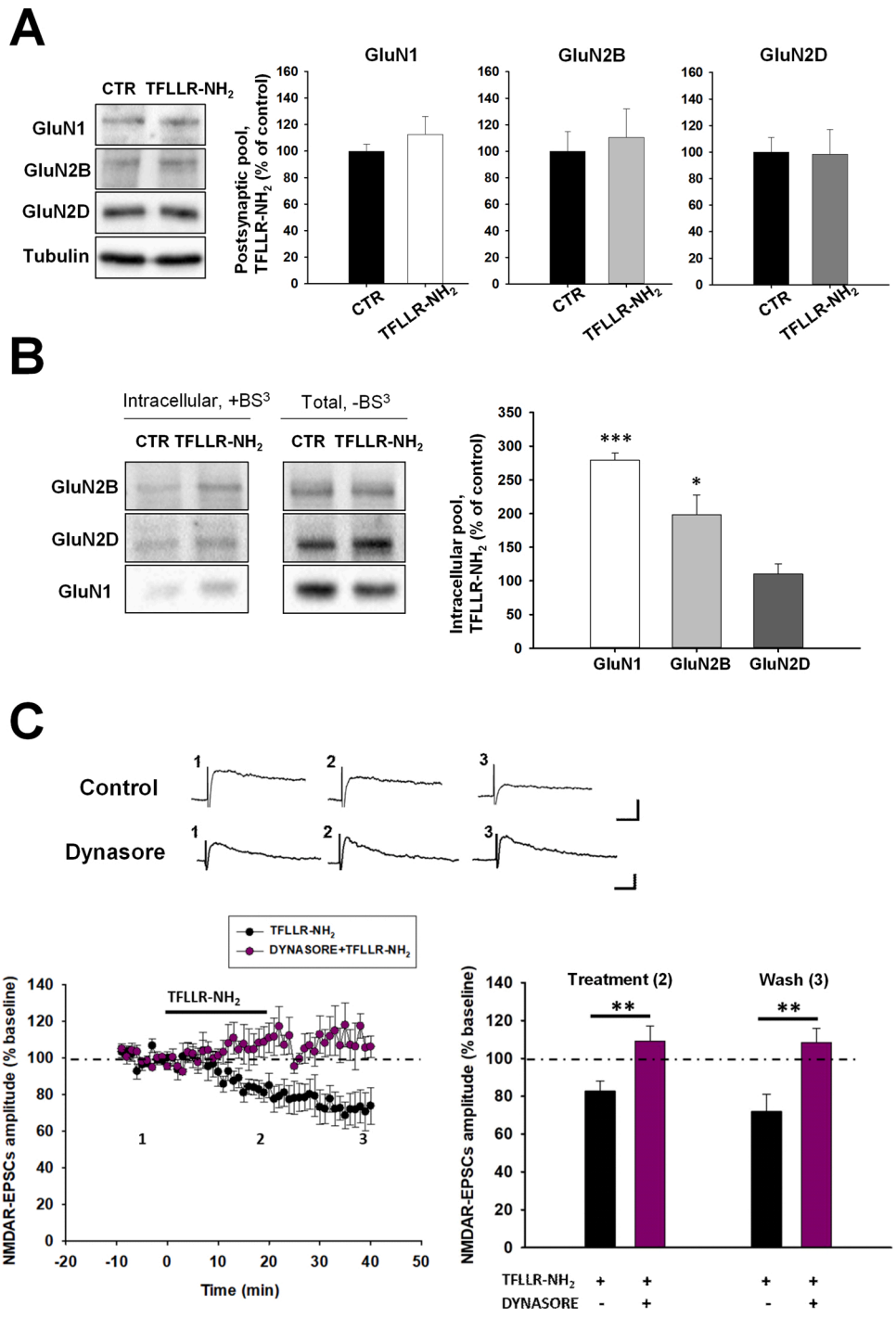

Fig. 5. PAR1 activation induces NMDARs internalization in nigral DA neurons.

A) Effect of PAR1 stimulation on NMDARs' composition in the postsynaptic density. Treatment with TFLLR-NH_2 does not alter total expression levels in the postsynaptic density of NMDARs subunits, GluN1, GluN2B and GluN2D with respect to control conditions, as demonstrated by WB analysis performed on postsynaptic TIF fraction of SN dissected from midbrain slices. For each protein, representative blots are shown with relative semiquantitative histogram. Protein levels are expressed as optical density (OD) normalized on tubulin levels and shown in the graph as percentage of control mean ± SEM. (n = 7, from 28 mice, unpaired *t*-test).

B) PAR1 stimulation induces increased NMDARs internalization in SNpc DAergic neurons. After treatment of SNpc-containing midbrain slices, NMDARs subunits GluN1, GluN2B, GluN2D expression in surface membrane was evaluated using the surface labeling assay with the cross-linker agent BS^3. As demonstrated by WB analysis, treatment with the PAR1 agonist TFLLR-NH_2 strongly increases intracellular levels of GluN1 and GluN2B subunits. Intracellular protein levels of GluN1, GluN2B, GluN2D subunits (+BS^3) were normalized on total cellular subunits levels (-BS^3). For each protein representative blot is shown with relative semiquantitative histogram. Protein levels are expressed as optical density (OD) and shown in the graph as percentage of control mean ± SEM. (n = 3, from 12 mice). * $p < 0.05$; ***$p < 0.0005$ TFLLR-NH_2 vs CTR, unpaired *t*-test.

C) Rescue effect of PAR1-dependent NMDARs hypofunction in SNpc DAergic neurons by preventing NMDARs endocytosis. A treatment with dynasore, an inhibitor of dynamin-dependent receptor internalization, counteracts PAR1-induced reduction of NMDAR-EPSCs in SNpc DAergic neurons, as demonstrated by representative traces of NMDAR-EPSCs (upper panel) recorded (1) before TFLLR-NH_2, (2) at 20 min of drug application, and (3) at 20 min after drug washout. Scale bar: 100 pA, 50 ms. Time-course plot of normalized NMDAR-EPSCs amplitude (left panel) and histogram of mean NMDAR-EPSCs amplitude (right panel) in TFLLR-NH_2- and dynasore + TFLLR-NH_2-treated slices demonstrate that dynasore completely antagonizes TFLLR-NH_2-induced NMDAR-EPSCs reduction. TFLLR-NH_2 (n = 10 cells/9 mice) and dynasore + TFLLR-NH_2 (n = 8 cells/6 mice). ** $p < 0.01$ TFLLR-NH_2 vs dynasore + TFLLR-NH_2, unpaired *t* test.

3.5. PAR1 activation induces NMDARs endocytosis in SNpc DAergic neurons

Despite NMDARs expression in postsynaptic fractions is unaltered, PAR1-dependent reduction of NMDAR-EPSCs in SNpc DAergic neurons might be due to a modification of local membrane trafficking and/or internalization events of NMDARs, which ultimately causes an alteration in the number of NMDARs expressed on the postsynaptic membrane of SNpc DAergic neurons.

To evaluate this hypothesis, we tested if NMDARs endocytosis could underlie TFLLR-NH_2-dependent inhibition of synaptic NMDAR in DAergic neurons. We quantified NMDARs expression in surface membrane of SNpc DAergic neurons, evaluating GluN1, GluN2B and GluN2D subunits levels by using the surface labeling assay with the cross-linker agent BS^3. We found that a treatment with the PAR1 agonist TFLLR-NH_2 strongly increased intracellular levels of GluN1 and GluN2B subunits (Fig. 5B). GluN2D intracellular levels were less modified upon TFLLR-NH_2 treatment (Fig. 5B).

To confirm that such PAR1-dependent NMDARs internalization is instrumental to NMDAR hypofunction observed in SNpc DAergic neurons upon PAR1 activation, we analyzed if TFLLR-NH_2-induced reduction of NMDAR-EPSCs could be counteracted by preventing NMDAR

endocytosis with dynasore, an inhibitor of dynamin-dependent receptors internalization. Interestingly, we found that a pretreatment with dynasore (80 μM) completely prevented PAR1-dependent NMDAR-EPSCs reduction (Fig. 5C). Indeed, TFLLR-NH_2 administration, in presence of dynasore, did not prompt any decrease in NMDAR-EPSCs amplitude in SNpc DA neurons, which appeared instead rather potentiated by the combined treatment with the PAR1 agonist and the dynamin inhibitor.

These results suggest that PAR1-induced synaptic NMDARs inhibition in SNpc DAergic neurons is reliant on a dynamin-dependent NMDARs endocytosis.

4. Discussion

Here we report that PAR1 stimulation causes a long-lasting reduction of synaptic NMDAR-mediated transmission in SNpc DAergic cells, which is dependent on PAR1-induced NMDARs internalization. Thus, we provide the evidence for a PAR1's function in the regulation of glutamatergic transmission in nigral DAergic neurons in physiological conditions.

4.1. PAR1-dependent regulation of NMDARs

PAR1 belongs to a distinctive family of GPCR, whose irreversible activation is endogenously achieved by a proteolytic cleavage by various serine proteases or MMPs. Differently from the previous believe about cerebral PAR1 activation only in pathological states by blood-derived ligand proteases which by-pass lesioned BBB, now it is well-accepted that PAR1 can be also activated in physiological conditions, thus regulating normal brain functions, including neurotransmission and synaptic plasticity [3,4].

Various groups have already demonstrated that serine proteases and matrix metalloproteases, as well as their zymogen precursors and endogenous inhibitors, can deeply influence synaptic functions in different brain areas, including hippocampus, striatum and cortex, thus modulating synaptic plasticity, as well as learning and memory processes, and complex behaviors [41–46]. A direct PAR1 activation might underlie such proteases-mediated effects, but the contribution of additional mechanisms due to direct proteolysis of extracellular matrix and synaptic molecules, or activation/inhibition of other proteases might also take a part.

Currently, little is known about selective PAR1 roles in the modulation of neurotransmission and synaptic plasticity, with evidence mainly restricted to the hippocampus. Indeed, PAR1 potentiates NMDARs function in hippocampal CA1 pyramidal neurons, by increasing glutamate release from astrocytes [47–49], and affect glutamatergic synaptic plasticity at CA3-CA1 synapses by fostering NMDAR-mediated long term potentiation (LTP) of field excitatory postsynaptic potentials (fEPSPs) [50]. Moreover, PAR1 activation in granule cells of the dentate gyrus increases neuronal excitability and potentiates an NMDAR-mediated component of fEPSPs in the dentate gyrus [51].

Contrariwise, other evidence documented PAR1-dependent inhibition of glutamatergic synaptic transmission in the hippocampus [52]. Indeed, stimulation of astrocytic PAR1, by speeding astrocyte-mediated extracellular glutamate clearance, reduces AMPARs and NMDARs functions in hippocampal CA1 pyramidal neurons, thus impairing LTP expression at Schaffer collateral synapses [52]. Thus, PAR1, through various mechanisms, differently affects glutamatergic synaptic transmission in the hippocampus.

Interestingly, here we show that in the SNpc PAR1 stimulation inhibits synaptic NMDARs in DAergic neurons, without affecting synaptic or extrasynaptic AMPARs. These results demonstrate that NMDARs are selectively involved in PAR1-dependent tuning of glutamatergic synaptic transmission in nigral DA neurons. This evidence further supports that PAR1 engages diverse and area-specific/cellular population-selective mechanisms to regulate neurotransmission and synaptic plasticity in various brain areas.

4.2. Cellular mechanisms underlying PAR1-induced NMDARs hypofunction

Studies exploring subunit composition of synaptic NMDARs in midbrain DA neurons support that GluN2B and GluN2D, but not GluN2A subunits, assembly to constitute functional synaptic NMDARs in nigral DA cells [39,40]. Indeed, NMDAR-EPSCs in nigral DA neurons are reduced by antagonists selective for GluN2B- and GluN2D subunits whereas they are mostly insensitive to GluN2A-selective antagonists [39,40], and, accordingly, mRNA and protein levels of GluN2B- and GluN2D subunits have been clearly reported in SNpc, while herein GluN2A levels are almost undetectable [39,40,53,54].

Our electrophysiological results indicate that NMDARs modulated by PAR1 comprise GluN2B- and GluN2D subunits. Actually, TFLLR-NH_2 reduced both GluN2B- and GluN2D-mediated components of EPSCs, as demonstrated by data obtained in the presence of selective antagonists. Magnitude of PAR1-induced reduction of GluN2B- or GluN2D-mediated component of EPSCs appears slightly different, being higher in the case of GluN2B-mediated currents. This might indicate that GluN2B subunits are a preferential (or more direct) target of PAR1-dependent pathways. In line with these results we found that PAR1 stimulation induces endocytosis of GluN2B and GluN1, as demonstrated by quantification of surface/intracellular NMDARs subunits obtained with the BS^3 cross-linker assay performed on midbrain slices. Notably, GluN2D subunits seem to be less internalized upon PAR1 stimulation, despite electrophysiological results support GluN2D contribution to PAR1-modulated NMDARs. Such apparent discrepancy might arise from possible different technical sensitivity of electrophysiological vs biochemical assays in detecting a significant GluN2D contribution. Nevertheless, our results clearly indicate that NMDARs endocytosis is the cellular mechanism by which PAR1 stimulation inhibits synaptic NMDARs in SNpc DA neurons. Indeed, TFLLR-NH_2-induced reduction of NMDAR-EPSCs in DA neurons is completely counteracted by a pretreatment with dynasore, a dynamin inhibitor, which prevents clathrin-dependent internalization of NMDARs.

Since PAR1 is coupled to various G proteins ($G_{q/11}$, $G_{i/o}$, and $G_{12/13}$) [2,3], multiple signaling pathways/molecular mechanisms downstream to its stimulation might cause NMDARs endocytosis in SNpc DA neurons. Actually, PAR1 can activate PLC and several kinases like PKC, PI3K, ERK, MAP, Src, and Rho, as well as can cause inhibition of AC [2,3], and most of these pathways, directly or indirectly, might regulate NMDARs trafficking. As membrane docking of NMDARs is tightly regulated by multiple phosphorylations/dephosphorylations occurring on GluN2B subunits [55] (the main target of PAR1's effects in our study), it is conceivable that PAR1, by regulating GluN2B phosphorylated ratio, might foster NMDARs endocytosis in SNpc DA neurons. However, specific pathways involved in such regulation remain to be determined. A PAR1-induced G_q-dependent PKC activation seems unlike to contribute to the TFLLR-NH_2-induced NMDARs inhibition, because the PKC-mediated GluN2B phosphorylation is usually associated to an increase in NMDARs surface levels, rather than an internalization. Similarly, the tyrosine kinases Src, which is also PAR1-activated, usually potentiates, rather than inhibits, NMDARs function [56]. A more likely mechanism by which PAR1 stimulation might cause NMDARs endocytosis in DA neurons is through G_i-dependent AC inhibition, which indirectly inhibits PKA by decreasing cAMP intracellular levels, because PKA, also by phosphorylating GluN2B, appears to foster NMDARs membrane stabilization [57].

4.3. PAR1 and midbrain dopaminergic system

Midbrain DAergic nuclei, SNpc and ventral tegmental area (VTA), are between brain areas with the highest expression of PAR1 [31,32,58].

Likewise, they show high levels of pro-thrombin, precursor of thrombin (the prototypical PAR1 activator) [31], thus indicating a preferential availability of endogenous PAR1 ligands in these brain areas to possible induce massive PAR1 activation in DAergic cells. In spite of this, the factual role of PAR1 in the regulation of midbrain DAergic neurons activity is still mysterious. Evidence for PAR1 effects in SNpc DAergic neurons in physiological conditions is limited to the observation that stimulation of PAR1 increases ERK phosphorylation in single DAergic neurons [22]. To date, PAR1 has been never reported to regulate neuronal excitability or synaptic transmission in nigral DAergic cells.

Differently, a potential contribution of PAR1 has been investigated in the context of neurodegeneration/neuroprotection of SNpc DAergic neurons [22,32–34], but with divergent results describing either a protective or a neurodegenerative role of PAR1 on DA neurons survival against neurotoxic insults in PD animal models. Indeed, in the MPTP model of PD, either genetic or pharmacological inhibition of PAR1 ameliorates MPTP-induced DA terminal damage, preserving DA content, tyrosine hydroxylase (TH) and dopamine transporter (DAT) levels, and reducing microgliosis [22], hence indicating that PAR1 exacerbates neurodegenerative processes inside DA neurons. Contrariwise, it has been reported that in a rat 6-OHDA-induced PD model, preconditioning with local infusion of a PAR1 agonist or thrombin, 3 days before 6-OHDA injection in medial forebrain bundle, ameliorates motor deficits, thus suggesting a protective role of PAR1 in counteracting the mechanisms underlying 6-OHDA-induced behavioral alterations [33].

Intracellular mechanisms underlying PAR1-mediated protective or detrimental effects on SNpc DAergic neurons are only partially elucidated. Among these it has been proposed that NMDARs activation is instrumental to PAR1-dependent neurodegeneration of SNpc DA neurons [34]. Interestingly, our results, showing a PAR1-dependent NMDA hypofunction in DA neurons may rather account for a neuroprotective mechanism driven by PAR1. Indeed, the PAR1-mediated inhibition of NMDARs functions in SNpc DA neurons could serve as a compensatory mechanism to counteract/neutralize aberrant glutamatergic inputs that arise from the subthalamic nucleus (STN) during basal ganglia dysfunctions associated with PD pathology [59], thus limiting DAergic neurons degeneration. Indeed, abnormal NMDARs activation, due to excessive glutamatergic inputs on nigral DA neurons, from the hyperactivated STN, is an overt mechanism fostering neuronal death of DA neurons in PD, mainly by prompting an abnormal Ca^{2+} influx that hyperactivate signaling pathways increasing reactive oxygen species (ROS) production and eliciting neurodegenerative processes [60]. Therefore, PAR1-induced NMDARs hypofunction, might contribute to preserve DA neurons survival from glutamate-mediated excitotoxicity, thus possible slowdown their progressive neurodegeneration in PD.

Regarding additional implications of PAR1-induced NMDAR hypofunction in nigral DA neurons, it should be considered that in midbrain DA neurons NMDARs are fundamental players in the induction of burst firing, by regulation of Ca^{2+} transients that initiate and terminate bursts via Ca^{2+}-activated potassium channels [61]. Since burst firing more efficiently increases extracellular DA in the projection areas, respect to regular firing, modifications in NMDARs-dependent bursts promotion might affect phasic DA release in the projection areas, that is involved in the encoding of salient stimuli, and thus critically influencing DA-dependent behaviors [62]. In this respect, PAR1-induced NMDARs hypofunction might affect transient extracellular DA release, by shifting threshold achievement for the occurrence of bursting events.

Moreover, NMDARs in midbrain DA cells are also instrumental for the induction of LTP of glutamatergic synaptic transmission [63,64]. Such NMDAR-dependent LTP in VTA DAergic neurons has been implied in the establishment of addictive-related behaviors. Thus, PAR1-dependent inhibition of synaptic NMDARs might affect such forms of synaptic plasticity in midbrain DA cells, and thus contributing to regulate DA-dependent functions and behaviors.

Interestingly, PAR1 roles in mesolimbic DA transmission have been analyzed in studies on animal models of dependence to nicotine and morphine. Actually, PAR1 activation regulates nicotine-induced DA release in nucleus accumbens (NAc) and modulates nicotine-induced conditioned place preference (CPP) and hyperlocomotion [58,65]. Moreover, PAR1 inhibition in the NAc, by means a local injection of a PAR1 antagonist, reduces morphine-induced DA release in the NAc and hyperlocomotion, but such treatment does not alter basal DA levels or spontaneous locomotor activity [66]. This evidence seems to support a PAR1 contribution to the regulation of DA outflow and DA-dependent behaviors (like locomotion) that is especially unmasked following drug of abuse/psychostimulants exposure, more than in basal conditions. Notably, despite this previous evidence provides interesting insights on the role of PAR1 in the regulations of mesolimbic DAergic system in vivo, future studies will be necessary to disentangle cellular loci and functional mechanisms underlying such PAR1-mediated effects.

5. Conclusions

Here we have described a novel role for PAR1 in the regulation of glutamatergic synaptic transmission in nigral DAergic neurons. To our awareness, this is the first PAR1-dependent mechanism regulating NMDARs function on these cells in physiological conditions. Future studies are required to translate the functional relevance of this modulation in SNpc DAergic cells in the regulation of DA-controlled functions, cognitive processes, and behaviors.

While factual PAR1 role in preserving nigral DAergic neurons survival in PD models remains yet debated, our data reveal a functional mechanism (i.e. NMDARs hypofunction in DA neurons) that could contribute to the PAR1-dependent neuroprotective effects on these cells.

Upcoming investigations are necessary to verify the precise role of the functional interaction between PAR1 and NMDARs in PD, as well as in other neurological and psychiatric diseases linked to DAergic system dysfunctions. Considering that aberrant activity of serine proteases and MMPs (PAR1 activators) has been reported in several neurological disorders including Alzheimer's disease, PD, TBI, stroke, epilepsy and familial encephalopathy with neuroserpin inclusion bodies (FENIB) [4,9,17,21,23,67–70], research efforts aimed to unravel PAR1-mediated functional mechanisms might result useful to a better definition of the etiology and treatment of diverse brain disorders.

Author contributions

R.P.: performed electrophysiological experiments; E.F.: performed biochemical experiments; F.G.: designed and supervised biochemical experiments; N.B.M.: conceived the study and designed electrophysiological experiments; A.L.: conceived the study and designed the experiments, performed collection of SN tissues for biochemical assays, supervised electrophysiological experiments, and wrote the manuscript.

All authors discussed the results and commented on the manuscript.

Funding

This work was supported by the Italian Ministry of Health (Ricerca Corrente to IRCCS Fondazione Santa Lucia).

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.105185.

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