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RESEARCH ARTICLE

Involvement of $P2Y_{12}$ receptors in a nitroglycerin-induced model of migraine in male mice

Flóra Gölöncsér[1] | Mária Baranyi[1] | András Iring[1] | László Hricisák[2] | Lilla Otrokocsi[1] | Zoltán Benyó[2] | Beáta Sperlágh[1,3]

[1]Laboratory of Molecular Pharmacology, Institute of Experimental Medicine, Budapest, Hungary

[2]Institute of Translational Medicine, Semmelweis University, Budapest, Hungary

[3]János Szentágothai Doctoral School, Semmelweis University, Budapest, Hungary

Correspondence
Beáta Sperlágh, Laboratory of Molecular Pharmacology, Institute of Experimental Medicine, Szigony u. 43, 1083 Budapest, Hungary.
Email: sperlagh@koki.mta.hu

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Background and Purpose: $P2Y_{12}$ receptors regulate different forms of pain and inflammation. In this study, we investigated the participation of $P2Y_{12}$ receptors in an animal model of migraine.

Experimental Approach: We tested the effect of the centrally administered selective $P2Y_{12}$ antagonist PSB-0739 and $P2Y_{12}$ receptor gene (*P2ry12*$^{-/-}$) deficiency in acute nitroglycerin-treated mice. Additionally, platelet depletion was used to investigate the role of platelet $P2Y_{12}$ receptors during migraine-like pain.

Key Results: Nitroglycerin induced sensory hypersensitivity of C57BL/6 wild-type (*P2ry12*$^{+/+}$) mice accompanied by an increase in c-fos and CGRP expression in the upper cervical spinal cord (C1–C2) and trigeminal nucleus caudalis. Similar changes were also observed in $P2Y_{12}$ gene-deficient (*P2ry12*$^{-/-}$) mice. Prophylactic intrathecal application of PSB-0739 reversed thermal hyperalgesia and head grooming time in wild-type mice but had no effect in *P2ry12*$^{-/-}$ mice. Furthermore, PSB-0739 was also effective when applied as a post-treatment. PSB-0739 administration suppressed the expression of c-fos in C1–C2 and trigeminal nucleus caudalis, and decreased the levels of dopamine and 5-hydroxytryptamine in C1-C2 in wild-type mice. Nitroglycerin treatment itself did not change adenosine diphosphate (ADP)-induced platelet activation measured by CD62P up-regulation in wild-type mice. Platelet depletion by anti-mouse CD41 antibody and clopidogrel attenuated nitroglycerin-induced thermal hypersensitivity and head grooming time in mice.

Conclusion and Implications: Our findings show that acute inhibition of $P2Y_{12}$ receptors alleviates migraine-like pain in mice by modulating the expression of c-fos and that platelet $P2Y_{12}$ receptors might contribute to this effect. Thus the blockade of $P2Y_{12}$ receptors may have therapeutic potential against migraine.

KEYWORDS
clopidogrel, migraine, mouse model, nitroglycerin, $P2Y_{12}$ receptor, PSB-0739

Abbreviations: PSB-0739, 1-amino-9,10-dihydro-9,10-dioxo-4-[[4-(phenylamino)-3-sulfophenyl]amino]-2-anthracenesulfonic acid sodium salt; TNC, trigeminal nucleus caudalis.

1 | INTRODUCTION

Migraine is one of the common pain disorders affecting the general population. Despite increasing knowledge regarding migraine pathophysiology, therapeutic strategies are limited and remain unsatisfactory for many patients (Dodick, 2018). According to one leading theory of migraine, central sensitisation in the trigeminal system involves the irritation of peripheral pain fibres and transient increase in the responsiveness of central neurons, which process information arising from intracranial structures and skin. During migraine attacks, the nociceptive signal is thought to be induced by the abnormally changed concentration of extracellular signalling molecules and neurotransmitters that are released from platelets, activated microglia and neurons (Andreou & Edvinsson, 2019).

Nucleotides participate in the pathophysiology of migraine, but their effects are contradictory depending on the type of P2 receptors (Burnstock, 2013). The purinergic signalling system plays an important role in the initiation and maintenance of migraine pain. **ATP** and **ADP** are involved in vasoconstriction and vasodilatation (Burnstock & Ralevic, 2014). Extensive evidence has shown the presence of **P2X** and **P2Y receptors** on neurons and satellite glial cells in the trigeminal ganglion (Haanes et al., 2019; Magni & Ceruti, 2013), as well as their participation in the nociception, confirming the role of endogenous nucleotides as potential triggers or mediators of migraine pain (Cieslak et al., 2015; Goloncser & Sperlagh, 2014; Jing et al., 2019; Long et al., 2018). Metabotropic **$P2Y_{12}$ receptors** expressed on platelets and microglia (Calovi et al., 2019; Cserep et al., 2020) are adenine nucleotide-preferring receptors, mainly responding to ADP, and couple to G_i, leading to a decrease in cAMP levels (Abbracchio et al., 2006). We and others have demonstrated that $P2Y_{12}$ receptor gene (*P2ry12*) deficiency and selective antagonists attenuate acute and chronic neuropathic and inflammatory pain (Horvath et al., 2014; S. Wang et al., 2018; Tatsumi et al., 2015; Tozaki-Saitoh et al., 2008), and platelet $P2Y_{12}$ receptors contribute to this effect (Beko et al., 2017). A recent study has also demonstrated that systemic inhibition of $P2Y_{12}$ receptors by **clopidogrel** and MRS2395, a putative $P2Y_{12}$ antagonist, decreases nitroglycerin-induced mechanical and thermal hypersensitivity in a chronic nitroglycerin-induced mouse model of migraine (Jing et al., 2019). However, previous *in vitro* studies questioned MRS2395 as a selective antagonist at recombinant $P2Y_{12}$ receptors (Horvath et al., 2014), and the exact involvement of $P2Y_{12}$ receptors in migraine model is still controversial.

Several studies have explored the potential association between platelet biology and migraine and concluded that some peculiar aspects of platelet metabolism may actively be involved in the pathogenesis of migraine (Danese et al., 2014). **5-HT** is actively taken up by platelets, stored and secreted upon activation and might contribute to vasoconstriction and hypersensitisation of neurons (Borgdorff & Tangelder, 2012; Offermanns, 2006). Platelet **NO** plays a modulatory role in migraine pathogenesis by the amplification of reactive vasodilatation (Reuter et al., 2001). Increased release of pro-inflammatory cytokines, such as IL-1, **IL-6**, **IL-8 (CXCL8)** and **TNF-α**, is observed after platelet activation (Slaba & Kubes, 2017) and contributes to local sterile inflammation and facilitates pain signals (Burstein et al., 2011; Yan et al., 2012). $P2Y_{12}$ antagonists are widely used in the clinical practice as antiplatelet agents, that is clopidogrel and **ticagrelor**, and have been reported to reduce migraine headache and were proposed as prophylactic agents in migraine therapies (Chambers et al., 2014; Reisman et al., 2018). In this study, we investigated the role of $P2Y_{12}$ receptors in an acute nitroglycerin-induced migraine animal model using $P2Y_{12}$ receptor gene (*P2ry12*)-deficient mice and the selective $P2Y_{12}$ receptor antagonist **PSB-0739**.

We examined the effect of genetic deletion and pharmacological blockade of $P2Y_{12}$ on migraine-related symptoms in mice as well as on the level of c-fos, calcitonine gene-related peptide (CGRP) and catechol and indole amines in migraine-related areas of the brain and spinal cord. We found that *P2ry12* gene deficiency had no effect, except for the level of CGRP after nitroglycerin treatment, whereas acute pharmacological blockade of $P2Y_{12}$ effectively alleviated the above parameters in wild-type animals, providing evidence for the involvement of $P2Y_{12}$ receptors in these effects.

What is already known

- P2X and P2Y purinoceptors regulate different aspects of migraine and the initiation of migraine attacks.
- Genetic deficiency and pharmacological inhibition of the $P2Y_{12}$ metabotropic receptor inhibit neuropathic and inflammatory pain.

What does this study add

- $P2Y_{12}$ antagonism but not gene-knockout inhibited nitroglycerin-induced hypersensitivity and upper spinal 5-HT and dopamine increases.
- Platelet depletion or clopidogrel (peripheral $P2Y_{12}$ antagonist) inhibited nitroglycerin-induced hypersensitivity and abolished by both.

What is the clinical significance

- $P2Y_{12}$ receptor is a potential, pharmacologically relevant target as an acute anti-migraine therapy.

2 | METHODS

2.1 | Animals

The experiments were performed on male wild-type (C57BL/6, *P2ry12*$^{+/+}$, RRID:IMSR_JAX:000664) and $P2Y_{12}$ recep[or

gene-deficient (*P2ry12*$^{-/-}$) mice (12 weeks old) weighing 25–30 g. B6;129-P2ry12^{tm1Dgen}/H knockout mice (Deltagen, San Mateo, CA, USA; EMMA, Cat# EM:02301, RRID:IMSR_EM:02301) were bred and genotyped as described in our previous studies (MGTU, IEM) (Beko et al., 2017; Horvath et al., 2014). Mice were housed under a 12-h light/dark cycle in a temperature-controlled (23 ± 2°C) and humidity-controlled room (60 ± 10%) with food and water available ad libitum. All studies *in vivo* were carried out during the light phase of the cycle. At the end of the experiments, mice were killed with with carbon dioxide (CO_2) or isoflurane, blood was collected and then the brain was removed following no perfusion or fixed by whole-body transcardial perfusion according to the requirements of the experiment.

Animal care and all experimental studies were approved by the local Animal Care Committee of the IEM HAS (PEI/001/775-6/2015) and followed the guidelines of Directive 2010/63/EU. The animals were treated humanely and all efforts were made to minimise animal suffering and reduce the numbers of experimental animals. Animal studies are also reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). The exact number of mice in each experimental group was provided in their respective figure legends. The sample size was calculated as described previously (Charan & Biswas, 2013). A pilot study was performed to measure the basic paw withdrawal threshold in grams of nitroglycerin- and vehicle-treated mice. We estimated sample size using G*Power 3.1.9.4 software (RRID:SCR_01372) (Student's *t*-test: a priori: compute required sample size; power: 0.8; α error probability: 0.05; effect size: 0.5; total sample size: 26; between vehicle and nitroglycerin treatment in *P2ry12*$^{+/+}$ mice). Mice were randomly assigned to experimental groups prior to the start of the experiment. After the behavioural experiment, these mice were randomly selected and used for immunohistochemistry, flow cytometry and HPLC measurements. The data acquisition and quantifications were performed by investigators blind to the experimental status of the subject.

2.2 | Materials

The following chemicals were used: the antianginal agent and vasodilator **nitroglycerin** (Nitro POHL®, G. Pohl Boskamp GmbH & Co. KG, Hohenlockstedt, Germany), the highly potent and selective $P2Y_{12}$ antagonist PSB-0739 (Tocris, Minneapolis, MN, USA, Cat# 3983) and the clinically used pro-drug $P2Y_{12}$ antagonist (+)—clopidogrel hydrogen sulfate (Tocris, Minneapolis, MN, USA, Cat# 1820). For immunohistochemistry, primary antibodies against $P2Y_{12}$ receptor (rabbit polyclonal, AnaSpec Inc., MA, USA, Cat# AS-55043A, RRID: AB_2298886), c-fos (guinea pig polyclonal, Synaptic Systems, Göttingen, Germany, Cat# 226004, RRID:AB_2619946), Iba1 (guinea pig polyclonal, Synaptic Systems, Göttingen, Germany, Cat# 234004, RRID:AB_2493179) and CGRP (sheep polyclonal, Enzo Life Sciences, Inc., NY, USA, Cat# BML-CA1137-0100, RRID:AB_11181019). Secondary antibodies Alexa Fluor 647 (Cat# 706-605-148, RRID: AB_2340476) and Alexa Fluor 594 (Cat# 713-585-147, RRID: AB_2340748) were purchased from Jackson ImmunoResearch Europe Ltd (Cambridgeshire, UK), Alexa Fluor 488 (Cat# A-11034, RRID: AB_2576217) was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Hoechst 33342 (Cat# 5117) was purchased from Tocris (Minneapolis, MN, USA). For FACS, anti-human/mouse CD62P (Psel.KO2.3; Cat# 12-0626-80, RRID:AB_1210863) and anti-mouse/rat CD42d-phycoerythrin (PE) antibody (Cat# 12-0421-82, RRID:AB_10804771) were purchased from eBioscience (San Diego, CA, USA), and anti-mouse CD41 (Cat# 553847, RRID:AB_395084) and rat IgG_1, κ isotype antibody (Cat# 553922, RRID:AB_479672) were purchased from BD Biosciences (San Jose, CA, USA). For anaesthesia and killing, ketamine (Calypsol 50-mg·kg^{-1} solution for injection) and xylazine (CP-Xylazine 2% injection) were from Gedeon Richter PLC (Budapest, Hungary) and CP-Pharma GmbH (Burgdorf, Germany), while isoflurane (AbbVie Inc., IL, USA, Cat# B506) was from Hungaropharma (Budapest, Hungary), respectively. Other materials used for experiments were obtained from general commercial resources and were of the highest grade.

2.3 | Drug administration

Nitroglycerin (Nitro POHL ingredients: 1-mg·ml^{-1} nitroglycerin, 49-mg·ml^{-1} glucose monohydrate, diluted hydrochloric acid and water for injection) and its vehicle (49 mg glucose monohydrate ml^{-1}) were administered intraperitoneally (450 µl per 30-g mouse); PSB-0739 was dissolved in physiological saline and administered intrathecally (5 µl per mouse); clopidogrel and its vehicle (2.5% DMSO and 7.5% PEG in water for injections, 300 µl per 30-g mouse) were administered intraperitoneally. All drug solutions were freshly prepared on the day of use. Dose of administration was acutely 15 mg·kg^{-1} for nitroglycerin, 0.3 and 0.03 mg·kg^{-1} for PSB-0739 and 10 mg·kg^{-1} for clopidogrel. The selection of dose and route of application was based on literature data (Goloncser & Sperlagh, 2014; Horvath et al., 2014). The time of administration of each drug in each model is described in the relevant section.

Intrathecal injection was performed following the method of Mestre et al. (1994): this method enables the injection of a drug directly to the CNS without damaging the spinal cord. Briefly, after a short exposure to isoflurane the animal was fixed by gripping appropriately and firmly on its pelvic girdle with a thumb on one side and forefinger/middle finger on the other side. The upper body of the animal was held gently with the palm. Then the injection site between the L5 and L6 vertebrae was indicated with a thumb or forefinger of the other hand. The needle was gently and vertically inserted in the intersection of indentation and the syringe was kept in a central sagittal plane. The angle was reduced slowly when it connected with the bone and then the needle was slipped into the intervertebral space. An evident sudden tail flick is a sign of successful entry into the intradural space. Then, the syringe was manually held in position for a few seconds and progressively removed to avoid any outflow of the drug. A 25 G × 5/8″ needle connected to a 250-µl Hamilton syringe with a repeating dispenser is suited for intrathecal delivery in mice. The

presence of PSB-0739 in the rostral spinal cord, trigeminal nucleus caudalis (TNC), somatosensory cortex (S1) and prefrontal cortex (PFC) after intrathecal delivery was verified by HPLC analysis (see in Table S1 and Figure S1).

2.4 | Femoral artery cannulation

In order to measure the effect of nitroglycerin on the systemic BP and heart rate (HR), a catheter was inserted into the left femoral artery, as described previously (Iring et al., 2017; Polycarpou et al., 2016). Briefly, animals were anaesthetised with 2% isoflurane during femoral artery catheterisation and subsequently with intraperitoneally applied ketamine (100 mg·kg^{-1}) and xylazine (10 mg·kg^{-1}) during BP measurement. The depth of the anaesthesia was frequently tested by checking the plantar nociception or corneal reflex, and for the maintenance of anaesthesia, additional anaesthetic was administered as necessary. The left femoral artery was cannulated under a stereomicroscope and it was used for continuous systemic arterial pressure measurement. Body temperature was maintained between 36°C and 37°C throughout the experiment by using a heating pad, controlled by a rectal probe. Nitroglycerin was applied intraperitoneally (15 mg·kg^{-1}). Arterial BP was measured and recorded continuously using the MP100 system and AcqKnowledge 3.72 software from Biopac Systems Inc. (Goleta, CA, USA, RRID:SCR_014279).

2.5 | Sensory sensitivity testing

In order to investigate the effect of nitroglycerin on the migraine-like symptoms in mice such as thermal hypersensitivity of the skin, light aversion and head-directed grooming, a hotplate test, head grooming test and light–dark box test were done. All behavioural experiments were carried out between 9:00 AM and 2:00 PM. Animals were habituated to the testing room for 1 h prior to behavioural tests.

2.5.1 | The hotplate test

To test the responsiveness of mice to nociceptive heat, stimulation was measured by an increasing-temperature hot/cold plate system (Ugo Basile, Gemonio, Italy, Cat# 35150). On the day of testing, animals were habituated to the testing apparatus for 10 min prior to the determination of the baseline nociceptive threshold. The animals were placed on an electrically heated metal plate that was kept at a constant temperature of 30°C (starting temperature). After the habituation period, the plate was heated from the starting temperature with a constant rate of 6°C·min^{-1} until the animals showed nocifensive behaviour (frequent paw lifting and/or paw licking in both front and back paws, jumping). Heating was then instantly stopped, the animal was removed from the apparatus and the plate was rapidly cooled. The temperature at which the animal showed the first sign of nocifensive behaviour was taken as the paw withdrawal threshold, expressed in °C. Approximately 1 h later, the measurement was repeated and the average of two values was taken as the baseline thermal nociceptive threshold. After the second measurement, the animals received treatment with drugs as described above, and 1 and 2 h after nitroglycerin administration, post-drug nociceptive threshold was measured.

2.5.2 | The head grooming test

For head pain, on the test day, after the 15-min habituation period, 1 and 2 h following systemic injection of vehicle or nitroglycerin, animals were placed in glass cylinder and their grooming behaviour was video recorded over 30 min with a video camera placed 50 cm in front of the cylinder. Videos were analysed using The Observer XT 13.0 software (Noldus Information Technology, Wageningen, the Netherlands, Cat# NDS-NSE-OBS-BASE). Head grooming behaviour was defined as the bilateral head/face cleaning movement with the forepaws or hind paws. Increasing time spent on head grooming was considered as an index of spontaneous facial pain (Chanda et al., 2013). Intrathecal PSB-0739 or its vehicle was given as indciated above and after the habituation period of 15 min the nitroglycerin was administered.

2.5.3 | The light-aversion test

For light sensitivity assay, light/dark boxes were used to quantify the photophobia associated with migraine (Markovics et al., 2012). Light-aversive behaviour was examined in mice at 1 h (60–80 min) and 2 h (120–140 min), in 20-min phases following administration of nitroglycerin. The mice were individually tested in the test apparatus consisting of four individual square chambers (L 40 cm × W 40 cm × H 25 cm for each). Each chamber was divided into two compartments of equal size, one white and one black. The white chamber without a top of the apparatus served as the light portion (400 lx) and the black chamber was fully enclosed and served as the dark portion (2 lx). A small opening (4 × 4 cm) connected the two compartments. Four chambers were used in parallel to test a vehicle- and nitroglycerin-treated mouse at the same time. On the test day, after 1-h conditioning period in the testing room, the animals were injected intraperitoneally with nitroglycerin or vehicle, and 1 and 2 h after the treatment mice were immediately placed into the centre of the white box. Time in the light chamber during 20-min sessions was recorded via a camera, which was set above the chambers. Following the 1-h time point observation, the mice were put back to their home cages and placed into the light–dark box again 120 min after treatment for a next observation session. The chambers were thoroughly cleaned and scrubbed with 70% ethanol between the testing of each animal. All experiments were performed between 9:00 AM and 2:00 PM. The videos of the experiments were recorded by Noldus MediaRecorder as digital video files. The position of the mice in white chamber was traced using EthoVision XT 13.0 (Noldus Information Technology,

Wageningen, the Netherlands, RRID:SCR_000441) to analyse the digital video files. The output parameters included duration in the light chamber, velocity and locomotion. The percentage of time spent in light chamber was calculated over the 20-min recording period. Intrathecal PSB-0739 or its vehicle was applied to the above-mentioned method 15 min prior to nitroglycerin treatment.

2.6 | Immunohistochemistry

In order to study whether nitroglycerin enhances the $P2Y_{12}$ receptor, c-fos and CGRP responses in the nervous system, immunofluorescence staining was carried out. After behavioural experiments, brains were taken out, mice were killed with CO_2 and then transcardially perfused with 10 ml of 0.9% saline, followed by 50 ml of 4% paraformaldehyde in PBS, and the whole brain and spinal cord were postfixed for 24 h in fixative at 4°C. Coronal sections (40 μm thick) from the medullary segment containing the TNC and upper cervical spinal cord (C1–C2) were cut by vibratome (Leica Microsystems GmbH, Germany). Every third section was blocked with 5% normal donkey serum containing 0.3% Triton X-100 for 1 h at room temperature and was then incubated with the following primary antibodies overnight at 4°C: rabbit anti-$P2Y_{12}$R (1:1000), guinea pig anti-c-fos (1:500) and sheep anti-CGRP (1:500). After washing in PBS, sections were incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor 488, Alexa Fluor 647 and Alexa Fluor 594, 1:500) and nuclei were stained with Hoechst 33342 (1:10,000) for 2 h at room temperature. Sections were mounted and images were acquired with a C2 confocal microscope (Nikon, Japan). The fluorescence signal intensity was quantified using image analysis software (NIS-Elements, Nikon, RRID: SCR_014329). To quantify c-fos-positive cell profiles and fluorescent intensity of $P2Y_{12}$ and CGRP in spinal C1–C2 and TNC, five to six mice were randomly selected from each treatment group. All immunoreactive cells in the one side of the C1–C2 and TNC area were counted manually and fluorescent intensity was calculated with ImageJ software (NIH, Bethesda, MD, USA, RRID:SCR_003070) by an investigator blinded to the treatments and calculated the average number of c-fos positive nuclei and fluorescent intensity in each section. The specificity of the $P2Y_{12}$ receptor antibody was confirmed by performing immunohistochemistry on the brain of *P2ry12*$^{-/-}$ mice. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.7 | HPLC determination of catechol and indole amine content

In order to measure the effect of nitroglycerin on the level of noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT; serotonin)) in the CNS, HPLC was used. Mice that received nitroglycerin and clopidogrel in vehicle or PSB or platelet depletion pretreatments were decapitated and the prefrontal cortex, the primary somatosensory cortex (S1) and the TNC areas of the brain and the C1–C2 part were dissected on ice and then were frozen by liquid nitrogen. The tissue was homogenised by ultrasonication in 200-μl volume of ice-cold 0.01-M perchloric acid solution that contained theophylline (as an internal standard) at 10-μM concentration and 0.5-mM sodium metabisulphite (antioxidant for biogenic amines). The tissue extract was centrifuged at 3510× *g* for 10 min at 0–4°C and the pellet was saved for protein measurement according to Lowry et al. (1951). Perchloric anion from the supernatant was precipitated by 1-M potassium hydroxide and the precipitate was then removed by centrifugation. The extracted monoamines were kept at −20°C until analysis.

Quantification of biogenic amines from tissue was performed by online column switching separation. The online solid-phase extraction (SPE) was carried out on an ACE UltraCore Super PhenylHexyl (7.5 cm × 2.1-mm I.D., 5-μm particle size) column and for separation, an ACE UltraCore Super C-18 (150 × 2.1-mm I.D., 5-μm particle size) analytical column from Advanced Chromatography Technologies Ltd (Aberdeen, Scotland) was used. The flow rate of the mobile phases ('A' 10-mM potassium phosphate and 0.25-mM EDTA; 'B' with 0.45-mM octane sulphonyl acid sodium salt, 8% acetonitrile [v/v] and 2% methanol [v/v], pH 5.2) was 350 or 450 μl·min^{-1}, respectively, in a step gradient application (Baranyi et al., 2006). The enrichment and stripping flow rate of buffer (10-mM potassium phosphate, pH 5.2) was during 4 min and the total runtime was 55 min.

The HPLC system used was a Shimadzu LC-20AD Analytical & Measuring Instruments System (Shimadzu Europa GmbH, Duisburg, Germany), with an Agilent 1100 Series Variable Wavelength Detector (Agilent Technologies, CA, USA) and BAS CC-4 amperometric detector (Bioanalytical Systems, Inc., IN, USA) in a cascade line. The detection of internal standard (theophylline) was performed at 253-nm wavelengths by UV and the biogenic amines at +0.73-V potential of electrochemical detection. Concentrations were calculated by a two-point calibration curve internal standard method: $(A_i \times f \times B)/(C \times D_i \times E)$, where A_i is the area of biogenic amine component, B is the sample volume, C is the injection volume, D_i is the response factor of 1-pmol biogenic amine standard, E is the protein content of sample and f is the factor of internal standard (IS area in calibration/IS area in actual). The data were expressed as pmol·mg^{-1} protein.

2.8 | Assessment of platelet CD62P levels by flow cytometry

In order to investigate how nitroglycerin altered platelet activation, we measured ADP-induced changes in platelet CD62P levels *ex vivo* in platelet-rich plasma samples, as described previously (Beko et al., 2017). Wild-type mice were treated with nitroglycerin (15 mg·kg^{-1}, i.p.), or its vehicle. Blood samples were taken directly from the vena cava of anaesthetised mice 120 min after the treatment. Apyrase (1 U·ml^{-1}) was added to the samples to prevent ADP receptor desensitisation. After 10 min of centrifugation at 200× *g*, platelet-rich plasma was collected. Platelet activation was induced by

ADP (250 μM, Sigma, MO, USA, Cat# A2754) and changes in platelet CD62P levels were assessed after 60 min of incubation. Platelets were stained with anti-human/mouse CD62P (Psel.KO2.3) antibody for 10 min. Samples were acquired with a BD FACSVerse machine and analysed with BD FACSUITE software (BD Biosciences, San Jose, CA, USA, RRID:SCR_013311). Changes in CD62P mean fluorescence intensity values were determined on CD42d-positive platelets.

2.9 | Platelet depletion

In order to assess the contribution of platelets in migraine-like symptoms *in vivo*, we ablated platelets with specific anti-**CD41** antibody as we have previously reported (Beko et al., 2017). Wild-type mice were injected intraperitoneally with purified rat anti-mouse CD41 for two consecutive days (25 μg in PBS, 200 μl per mouse). As a control, we used purified rat IgG_1, κ isotype antibody (25 μg in PBS, 200 μl per mouse). Platelet depletion was confirmed by flow cytometry with an anti-mouse/rat CD42d-PE antibody on a BD FACSVerse instrument. Blood samples were taken from the tail vein (conscious mice) before and 24 h after the first CD41/IgG1 treatment or vena cava (anaesthetized mice) 24 h after second CD41/IgG_1 treatment and after the hotplate test, with acid citrate dextrose solution (ACD) as an anticoagulant.

2.10 | Statistics

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). We calculated the number of animals and group sizes to be used via G*Power 3.1.9.4 software and also based on our previous experience. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$. We note that group size is the number of independent values. The outliers were included in data analysis and presentation. All values are presented as the mean ± SEM (error bars). The normality of experimental data distribution was tested by the Shapiro–Wilk normality test. Depending on the data sets, statistical analyses were performed using unpaired Student's *t*-test, one-way ANOVAs with Tukey's post hoc test or two-way ANOVAs with Tukey's post hoc test with or without repeated measures using STATISTICA Version 13.5.0.17 software (TIBCO Software Inc., Palo Alto, CA, USA, RRID:SCR_014213). Post hoc tests were conducted only if F in ANOVA achieved $P < 0.05$. P values of less than 0.05 were considered statistically significant throughout the study.

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Nitroglycerin treatment transiently decreases mean arterial pressure and increases HR, but has limited influence on migraine-associated symptoms at 60 min

Basal mean arterial pressure (MAP) and HR were within the physiological range for both genotypes and baseline MAP and HR were also similar in the anaesthetised *P2ry12*$^{+/+}$ (86.2 ± 2.6 mmHg and 296 ± 17 BPM, respectively) and *P2ry12*$^{-/-}$ (76.5 ± 2.5 mmHg and 277 ± 15 BPM, respectively) mice (Figure 1). Intraperitoneal administration of 15-mg·kg^{-1} nitroglycerin induced a marked, but a transient reduction by 40% (in WT) and 34% (in KO) in MAP (to 51.5 ± 3.1 mmHg in WT and 50.9 ± 4.3 mmHg in KO at 1 min) (Figure 1a), accompanied by a transient increase by 32% (in WT) and 24% (in KO) in HR (387 ± 18 BPM in WT and 342 ± 19 BPM in KO at 1 min) (Figure 1b) compared with baseline conditions. Within minutes, both MAP and HR started to normalise and returned close to basal values. Importantly, hypotension was transient and the MAP returned to the range of cerebral autoregulation within approximately 5 min. One hour after nitroglycerin application, when our migraine experiments were performed, both MAP (76.0 ± 3.5 mmHg in WT and 74.2 ± 3.1 mmHg in KO) and HR (282 ± 26 BPM in WT and 266 ± 22 BPM in KO) had returned to near basal levels (Figure 1a,b). Furthermore, neither MAP nor HR were significantly different between wild-type and knockout mice during the measurement. Based on these observations, we can conclude that the effect of nitroglycerin on the systemic circulation after 1 h is negligible and has a limited effect in our experiments on studying the involvement of $P2Y_{12}$ receptors in migraine.

3.2 | Inhibition of $P2Y_{12}$ receptor relieves migraine-associated symptoms in nitroglycerin-treated mice

Consistent with previous studies, nitroglycerin treatment decreased the paw withdrawal threshold to noxious heat in a time-dependent manner when compared with both before and 2 h after nitroglycerin administration. The baseline nociceptive threshold was not significantly different between wild-type and *P2ry12*$^{-/-}$ mice (see appropriate baseline paw withdrawal threshold values of the groups in Table S2). Nitroglycerin significantly reduced paw withdrawal threshold, when compared with vehicle treatment in both genotypes (Figure 2a). Nevertheless, the nitroglycerin-induced decrease in paw withdrawal threshold was not significantly different between *P2ry12*$^{+/+}$ and *P2ry12*$^{-/-}$ mice (Figure 2a). We also examined mechanical hyperalgesia in wild-type mice, but acute nitroglycerin did

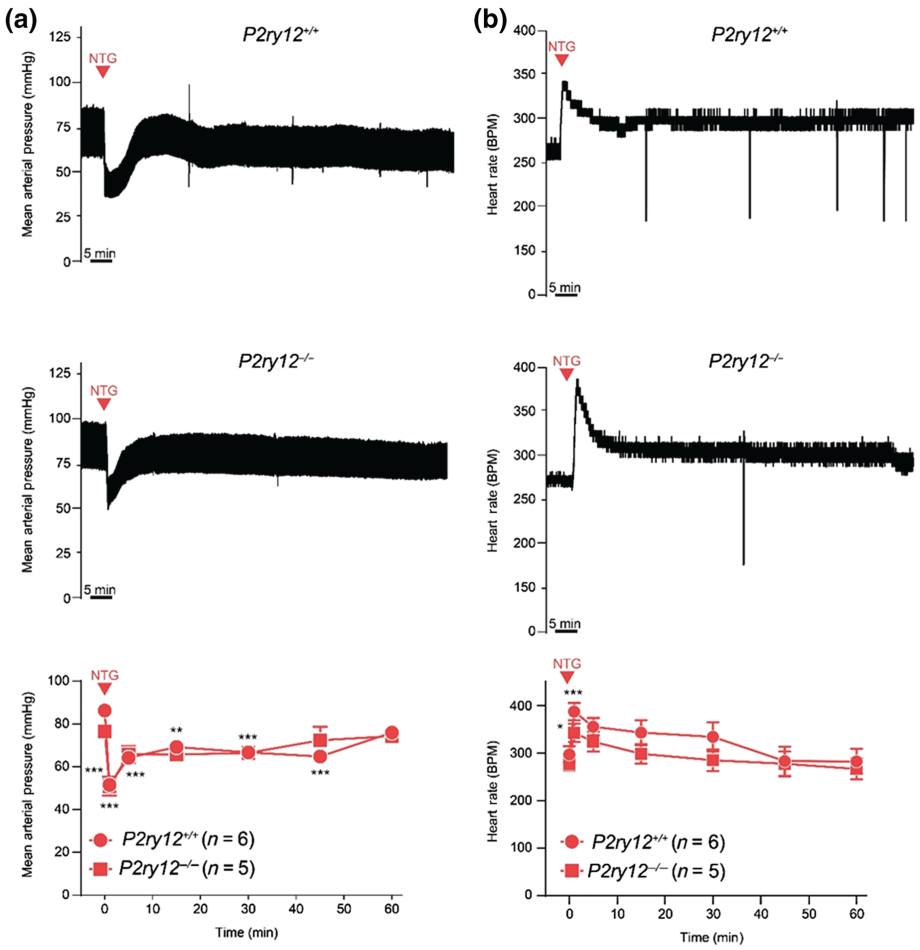

FIGURE 1 Effect of nitroglycerin (NTG) administration on mean arterial pressure (a) and heart rate (b). Representative recordings in *P2ry12*$^{+/+}$ and *P2ry12*$^{-/-}$ animals' mean arterial pressure (*left panels*) or heart rate (*right panels*) and averaged results of multiple experiments in wild-type and *P2ry12*$^{-/-}$ mice. Administration of NTG (15 mg·kg^{-1}, i.p.) is indicated by arrows. Physiological parameters were determined before (0 min) as well as at 1, 5, 15, 30, 45 and 60 min after NTG for statistical analysis (*lower panels*). (a) NTG treatment significantly decreases MAP in both genotypes (time: $F_{6, 54} = 20.604$, $P < 0.05$; genotype: $F_{1, 9} = 0.007$, $P > 0.05$; and genotype × time: $F_{6, 54} = 1.851$, $P > 0.05$). (b) NTG treatment causes increased HR in both genotypes (time: $F_{6, 54} = 12.0517$, $P < 0.05$; genotype: $F_{1, 9} = 1.3274$, $P < 0.05$; and genotype × time: $F_{6, 54} = 1.0320$, $P > 0.05$). Values are presented as mean ± SEM; $^{*}P < 0.05$ compared with baseline values of the same treatment group (repeated measures ANOVA with Tukey's post hoc test [a, b])

not produce significant changes in paw withdrawal threshold of mice (Figure S2).

To evaluate whether acute inhibition of $P2Y_{12}$ receptor is able to modify nitroglycerin-induced nociceptive behaviour, at first, we injected the $P2Y_{12}$ selective antagonist PSB-0739 (0.03 and 0.3 mg·kg^{-1}, i.t.) as a prophylactic agent upon single application 15 min before administration of nitroglycerin. We found that pre-treatment with PSB-0739 completely prevented the effect of nitroglycerin-induced thermal hypersensitivity in wild-type mice (Figure 2b). In contrast, PSB-0739 administration was ineffective in nitroglycerin-treated *P2ry12*$^{-/-}$ mice (Figure 2b). The effect of PSB-0739 was dose dependent, as a lower dose (0.03 mg·kg^{-1}, i.t.) elicited a smaller effect (Figure 2c). Additionally, PSB-0739 administered 30 min after the nitroglycerin injection likewise alleviated the nitroglycerin-induced thermal hypersensitivity in wild-type mice (Figure 2d). The peak effect of nitroglycerin was appeared at 30 min immediately before PSB-0739 intrathecal injection. Paw withdrawal threshold in saline-treated group was lower at 1 h compared with PSB-0739-treated group (Figure 2d). However, the effect of PSB-0739 given as a prophylactic agent appeared more effective compared with when it was applied following nitroglycerin.

We also showed that nitroglycerin significantly increased head grooming time as compared with the vehicle-treated group and this head grooming time was significantly different in wild-type and *P2ry12*$^{-/-}$ mice (Figure 3a,b). Furthermore, *P2ry12* gene deficiency did not attenuated head grooming time in the NTG-treated mice (Figure 3a,b), similar to thermal hyperalgesia in hot plate test. While PSB-0739 significantly decreased head grooming time in wild-type mice (Figure 3c,d).

The testing of the light-aversive behaviour of mice began 1 and 2 h after vehicle or nitroglycerin injection. However, nitroglycerin did not generate any light-aversive response in wild-type and *P2ry12*$^{-/-}$ mice (Figure 3e,f). When we looked at light aversion as a function of time over the 20-min testing period, there was no significant PSB-0739 pretreatment effect (Figure 3g). PSB-0739 did not influence the average time spent in light in both sessions in wild-type mice after nitroglycerin treatment (Figure 3h).

3.3 | Changes in $P2Y_{12}$ receptor, c-fos and CGRP immunoreactivity in the C1–C2 and trigeminal nucleus caudalis (TNC) after acute nitroglycerin administration

To investigate the effect of nitroglycerin treatment on $P2Y_{12}$ receptor, c-fos and CGRP expression, we performed immunofluorescence using specific antibodies (Figures 4 and 5). $P2Y_{12}$ receptor immunoreactivity

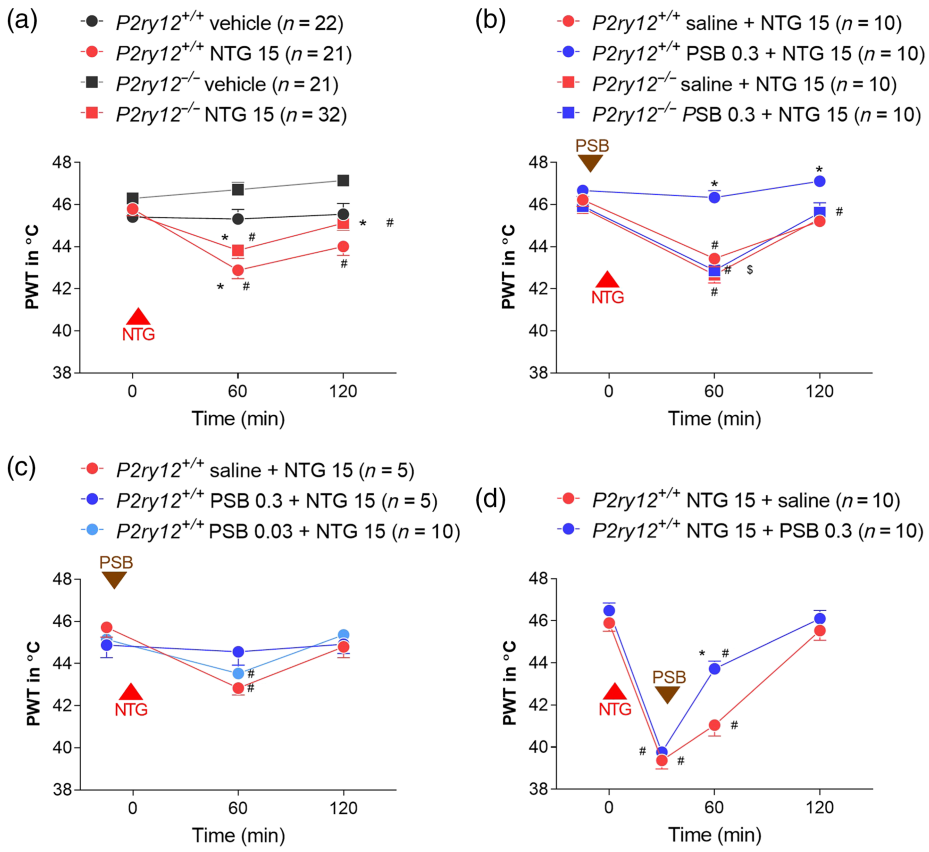

FIGURE 2 Effect of nitroglycerin (NTG) administration on heat sensitivity of mice. NTG induces thermal hypersensitivity in mice, which is reversed by the $P2Y_{12}$ receptor antagonist PSB-0739 in *P2ry12*$^{+/+}$, but not in *P2ry12*$^{-/-}$ mice. Administration of NTG (15 mg·kg^{-1}, i.p.) is indicated by red arrows and PSB-0739 and saline are pointed by brown arrows. (a) NTG treatment causes thermal hyperalgesia in mice (treatment effect: $F_{1, 92} = 31.9$, $P < 0.05$; treatment × genotype effect: $F_{1, 92} = 1.6$, $P > 0.05$). (b, c) Both doses of PSB-0739 (i.t.) are effective in *P2ry12*$^{+/+}$ mice and entirely inhibited NTG-induced thermal hypersensitivity, but not in *P2ry12*$^{-/-}$ mice (b) (treatment × genotype effect: $F_{1, 36} = 16.0$, $P < 0.05$). (d) Post-PSB-0739 treatment alleviates NTG-induced thermal hypersensitivity in *P2ry12*$^{+/+}$ mice (treatment effect: $F_{1, 18} = 9.51$, $P < 0.05$). The changes in nociceptive threshold after intraperitoneal NTG treatment are presented on the graph, as paw withdrawal threshold (PWT), expressed in °C. Values are presented as means ± SEM; $^{*}P < 0.05$ compared with control group (vehicle/saline treated) at the same time point, $^{\#}P < 0.05$ compared with baseline PWT values of the same treatment group and $^{\$}P < 0.05$ compared with *P2ry12*$^{-/-}$ mice at the same time point (repeated measures ANOVA with Tukey's post hoc test [a–d])

was mostly associated with microglia-like cells consistent with its predominant localisation in the CNS (Figure 4a) (Butovsky et al., 2014; Cserep et al., 2020). The difference in fluorescence intensity of $P2Y_{12}$ receptor in the C1–C2 and TNC regions of wild-type mice between nitroglycerin- and vehicle-treated group was unchanged (Figure 4b).

The number of c-fos-positive cells in the C1–C2 and TNC of wild-type and C1–C2 of *P2ry12*$^{-/-}$ mice was significantly increased 2 h after nitroglycerin injection (Figure 5a). We also observed that the fluorescence intensity of CGRP-immunoreactive fibres was also significantly increased by nitroglycerin administration in the C1–C2 and TNC of wild-type mice, but not in *P2ry12*$^{-/-}$ mice (Figure 5c).

As an alternative approach to determine, whether endogenous $P2Y_{12}$ receptor activity affects c-fos and CGRP expression, we acutely blocked $P2Y_{12}$ receptor using PSB-0739 (0.3 mg·kg^{-1}, i.t.). The results showed that the number of c-fos immunoreactive cells in the C1–C2 and TNC was significantly decreased in the group treated with PSB-0739 compared with the corresponding number in the saline + nitroglycerin-treated group (Figure 5d). The density of CGRP-immunoreactive fibres in the C1–C2 and TNC was not significantly changed after PSB-0739 treatment (Figure 5e).

3.4 | The effect of $P2Y_{12}$ receptor inhibition on nitroglycerin-induced changes in neurotransmitter levels in migraine-related areas of the CNS

To evaluate the effect of nitroglycerin on the neurotransmitter levels in migraine- and pain-related areas of the CNS, the level of noradrenaline, dopamine and 5-HT in mouse C1–C2, TNC, the somatosensory cortex (S1) and the prefrontal cortex was examined. PSB-0739 (0.3 mg·kg^{-1}, i.t.) or saline in an identical route and volume was applied as a pretreatment 15 min before respective nitroglycerin/vehicle treatment. As shown in Figure 6, nitroglycerin induced a significant elevation in the level of dopamine and 5-HT in the C1–C2 region of the spinal cord, when compared with vehicle treatment (Figure 6b, c), which could be prevented by PSB-0739 pretreatment. Nitroglycerin also elicited a significant increase in dopamine level in the

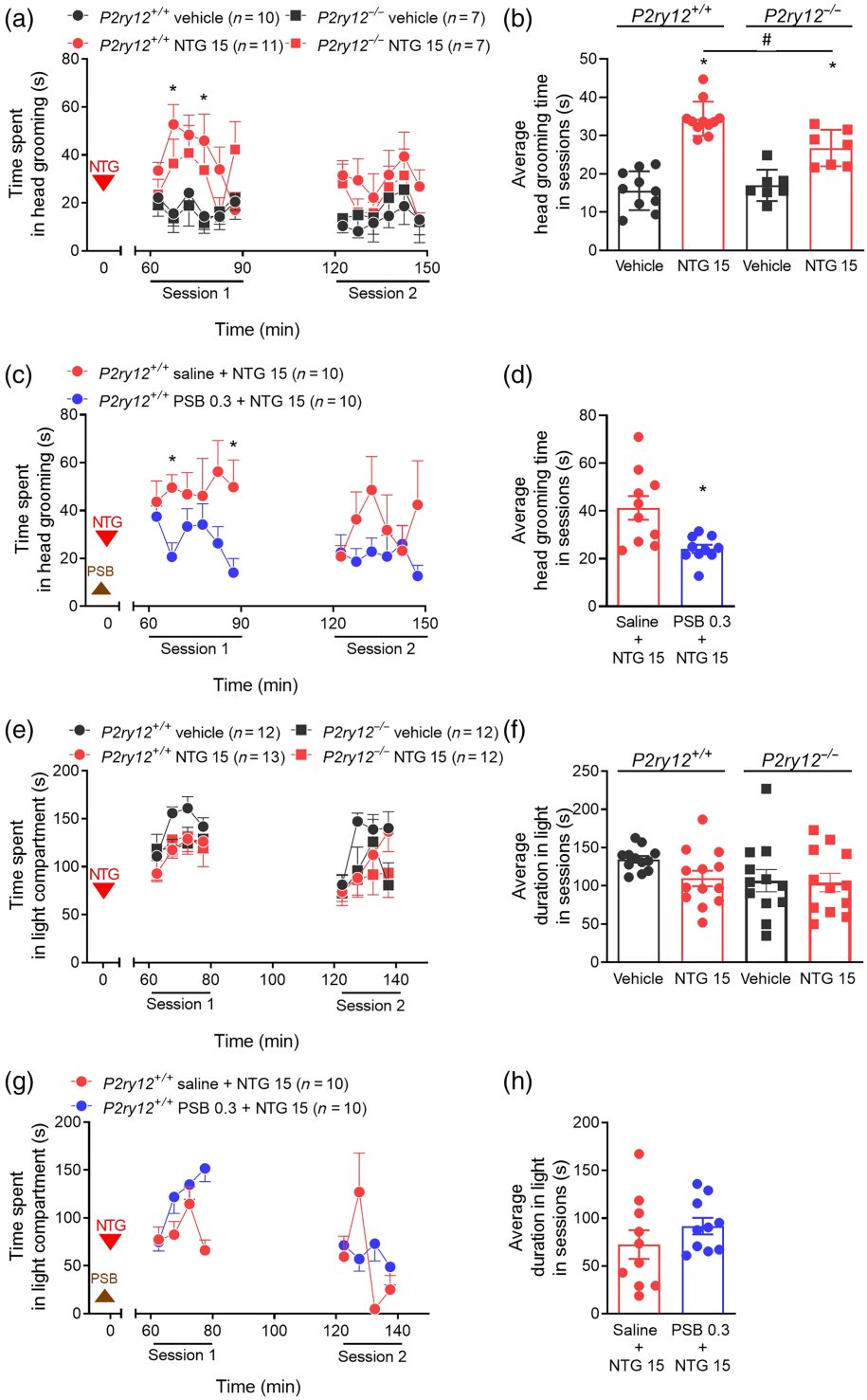

FIGURE 3 Effect of nitroglycerin (NTG) administration on head grooming time and light-aversion behaviour. Administration of NTG (15 mg·kg^{-1}, i.p.) is indicated by arrows and PSB-0739 and saline are pointed by brown arrows. (a–d) NTG treatment causes greater head grooming activity in wild-type and *P2ry12*$^{-/-}$ mice, but PSB-0739 (i.t.) is effective in alleviating this in *P2ry12*$^{+/+}$ mice. (a, c) The time spent in head grooming was recorded in the two 30-min test sessions and divided into 5-min intervals. (b) NTG treatment increases head grooming time in wild-type and *P2ry12*$^{-/-}$ mice (treatment effect: $F_{1, 31} = 79.043$, $P < 0.05$). (d) PSB-0739 treatment decreases the average head grooming time in wild-type mice ($P < 0.05$). (e–h) NTG treatment does not cause light-aversion behaviour in wild-type and *P2ry12*$^{-/-}$ mice and PSB-0739 has no effect either. (e, g) The time spent in light was recorded in 5-min intervals over the two 20-min test session. (f) NTG treatment did not influence the time spent in the light of mice (treatment effect: $F_{1, 45} = 1.5916$, $P > 0.05$; genotype effect: $F_{1, 45} = 2.3113$, $P > 0.05$). (g) Light aversion was unaffected by PSB-0739 in wild-type mice as a function of time (time × pretreatment effect: $F_{7, 126} = 7.2089$, $P > 0.05$). (h) PSB-0739 treatment did not influence the average time spent in light of mice in both sessions ($P > 0.05$). Values are presented as means ± SEM; $^{*}P < 0.05$ compared with control group (vehicle/saline treated) and $^{\#}P < 0.05$ compared with head grooming time of the same treatment group (Friedman test with Wilcoxon matched-pairs test [a, c]; two-way ANOVA with Tukey's post hoc test [b, f]; unpaired two-tailed Student's *t*-test [d, h]; and repeated measures ANOVA with Tukey's post hoc test [e, g])

prefrontal cortex and this was attenuated by PSB-0739 (Figure 6k). In contrast, nitroglycerin induced a decrease in noradrenaline content in the TNC and prefrontal cortex (Figure 6d,j) and dopamine content in the TNC (Figure 6e) and an increase of dopamine in the S1 region of somatosensory cortex (Figure 6h) that were not affected by the PSB-0739 pretreatment. These results indicate that nitroglycerin-induced changes in monoamine neurotransmitter levels in migraine-related CNS areas are modified by blockade of $P2Y_{12}$ receptor and these changes are region and neurotransmitter dependent.

3.5 | The effect of nitroglycerin on platelet activation

To assess the role of peripherally expressed $P2Y_{12}$ receptor, mainly located on platelets, first, we have tested nitroglycerin-induced platelet activation by measuring changes in CD62P expression in platelet-rich plasm derived from saline- and nitroglycerin-treated mice challenged by ADP *ex vivo* (Figure 7). ADP (250 μM) induced significant up-regulation of CD62P on CD42d-positive platelets (Figure 7d).

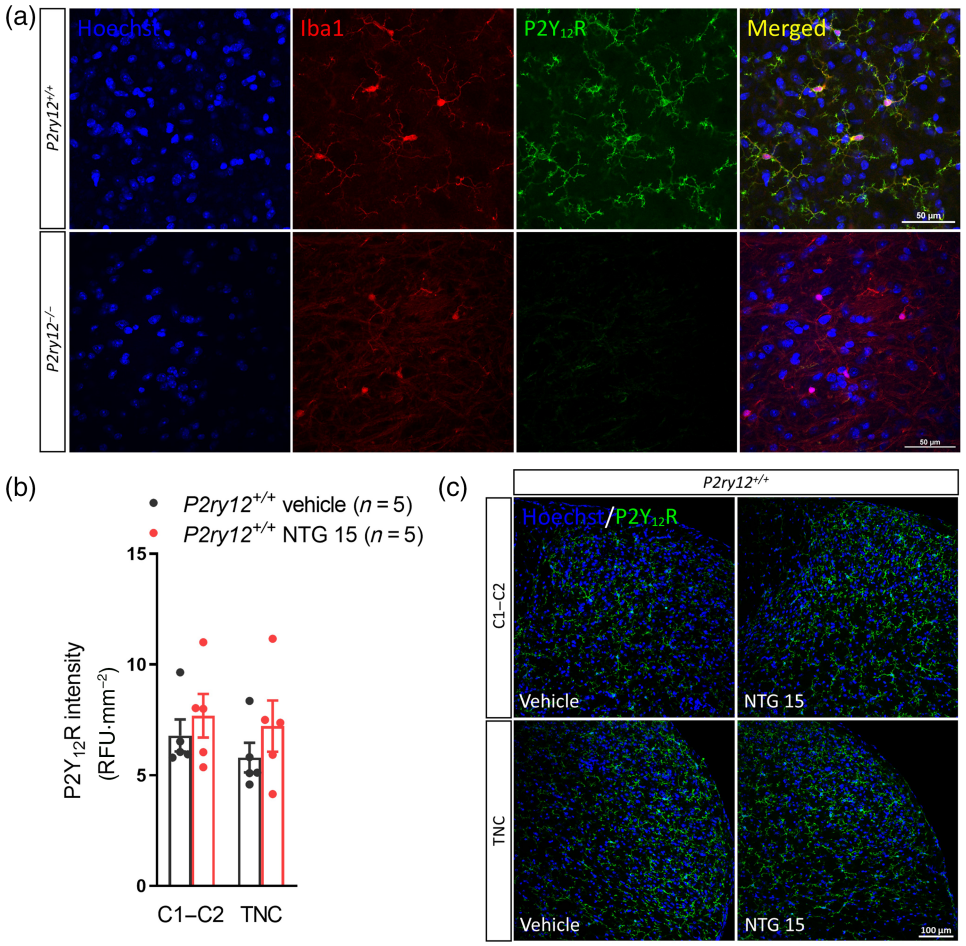

FIGURE 4 Effect of nitroglycerin (NTG) administration on $P2Y_{12}$ receptor (R) expression in *P2ry12*$^{+/+}$ mice. (a) Validation of the $P2Y_{12}$ receptor antibody specificity. Representative multi-immunofluorescence labelling images of trigeminal nucleus caudalis (TNC) from vehicle-treated *P2ry12*$^{+/+}$ and *P2ry12*$^{-/-}$ mice stained with antibodies directed against cell nuclei (Hoechst, blue), microglia cells (Iba1, red), $P2Y_{12}$ receptor (green) and overlay image (merged). Scale bar: 50 µm. (b) Quantitative analysis of the $P2Y_{12}$ receptor immunoreactive area in spinal C1–C2 and TNC after NTG injection (C1–C2: $P > 0.05$; TNC: $P > 0.05$; unpaired two-tailed Student's *t*-test). Images were analysed and background subtracted in ImageJ. To measure fluorescent intensity, a region of interest was drawn and the average signal was measured. (c) Representative immunofluorescence images of $P2Y_{12}$ receptor in the C1–C2 and TNC after NTG administration. Scale bar: 100 µm. Values are presented as mean ± SEM

nitroglycerin had no effect on ADP-induced platelet activation as compared with respective control (Figure 7d).

3.6 | Clopidogrel and platelet depletion protect mice from nitroglycerin-induced thermal hypersensitivity

Because we have observed no difference in platelet activation in nitroglycerin-treated mice, we have further investigated whether $P2Y_{12}$-driven actions in nitroglycerin-induced alteration in thermal hypersensitivity require the presence of functional platelets. For this purpose, mice were treated with anti-mouse CD41 antibody (25 µg, i.p.) two times before the nitroglycerin-induced heat sensitivity (hotplate) and head grooming tests, which resulted in depletion of the majority of platelets on the test day (decreased by 66.3%, Figure 8a, b). Leukocyte populations were not affected by the antibody treatment. The experimental design and timeline are shown in Figure 8b and clopidogrel (10 mg·kg^{-1}) and its vehicle were administered on the second day after baseline paw withdrawal threshold measurement. On test day, the platelet depletion did not affect the baseline threshold of thermal hyperalgesia in either group (see in Table S2). However, after the nitroglycerin treatment, the paw withdrawal threshold value was decreased in the control group (IgG_1-treated) but not in anti-mouse-CD41-treated mice (Figure 9a), indicating that platelets or mediators released from them contribute to nitroglycerin-induced hyperalgesia. Additionally, we examined nitroglycerin-induced head grooming in platelet-depleted mice (Figure 9b,c). Platelet depletion significantly decreased the total duration of grooming time. To evaluate the effect of acute, peripheral $P2Y_{12}$ receptor blockade on the hotplate test, the non-selective $P2Y_{12}$ receptor antagonist clopidogrel was applied intraperitoneally, because clopidogrel has only a limited penetration across the blood–brain barrier. Similarly to the effect of platelet depletion, clopidogrel also significantly attenuated nitroglycerin-induced hyperalgesia in non-depleted control mice (Figure 9d,e). When we applied platelet depletion and clopidogrel treatment together, the nitroglycerin-induced hyperalgesia was almost completely abolished (Figure 9d,e). Clopidogrel, platelet depletion and the combination of the two also partly replicated the effect of central $P2Y_{12}$ receptor blockade by PSB-0739 on nitroglycerin-induced changes in neurotransmitter levels in migraine-related areas of the CNS (Figure S5). These data indicate that endogenous $P2Y_{12}$ receptor activation and other substances released from platelets contribute to the development of migraine-like hypersensitivity.

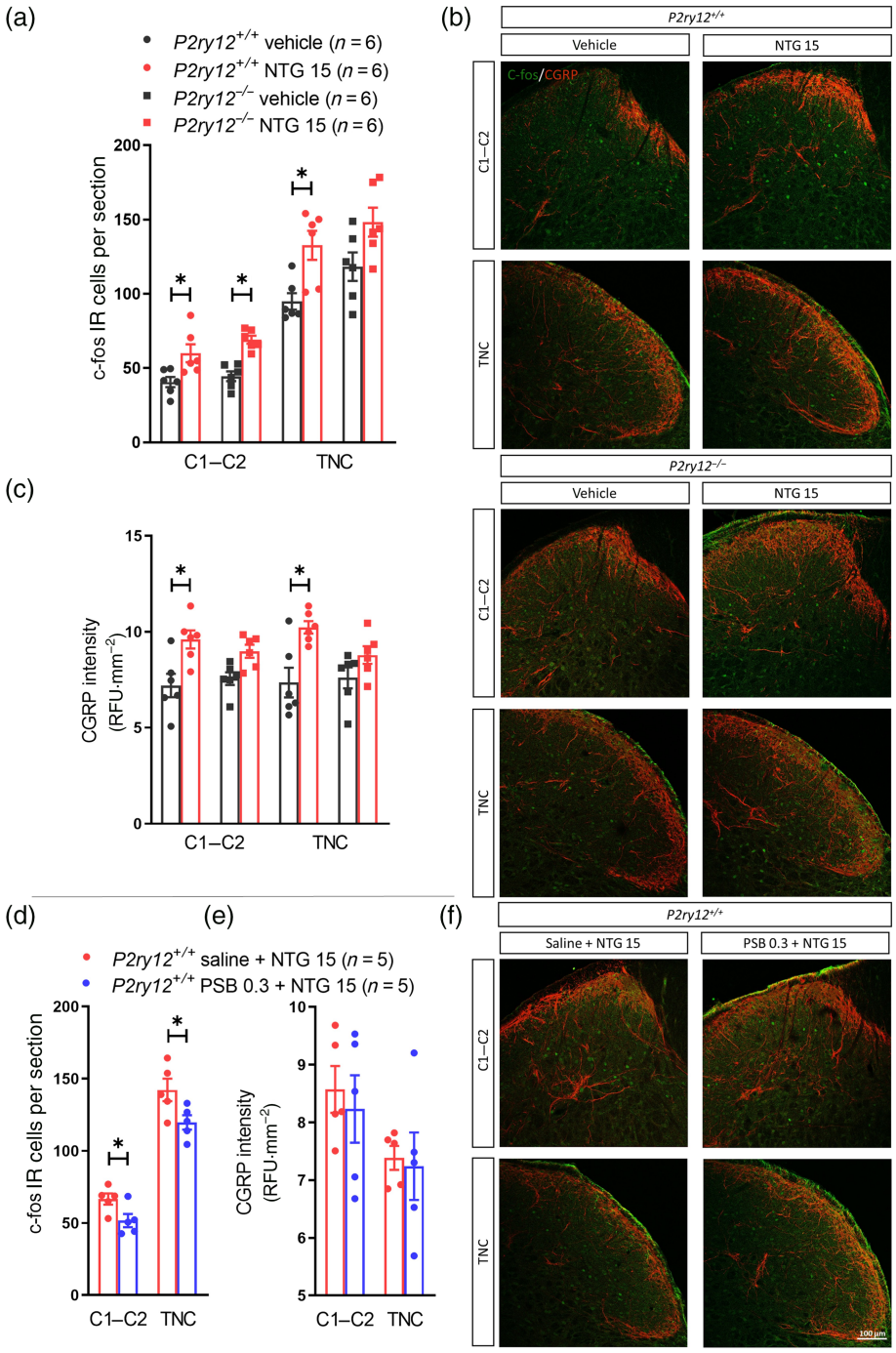

FIGURE 5 Effect of nitroglycerin (NTG) administration on c-fos and CGRP expression. NTG increases c-fos and CGRP expression in spinal C1–C2 and trigeminal nucleus caudalis (TNC) of *P2ry12*$^{+/+}$ and c-fos expression in the C1–C2 of *P2ry12*$^{-/-}$ mice. Inhibition of $P2Y_{12}$ receptor function reduces NTG-induced c-fos expression in the C1–C2 and TNC. Quantitative analysis of c-fos-IR cells (a) and CGRP fluorescence intensity (c) in the C1–C2 and TNC. (a) The number of c-fos-IR cells is significantly increased 2 h after NTG injection in C1–C2 and TNC of wild-type and C1–C2 of *P2ry12*$^{-/-}$ mice (C1–C2: treatment effect: $F_{1,\ 20} = 28.9895$, $P < 0.05$; TNC: treatment effect: $F_{1,\ 20} = 14.5880$, $P < 0.05$). (b) Representative multi-immunofluorescence labelling images of C1–C2 and TNC from vehicle- and NTG-treated *P2ry12*$^{+/+}$ and *P2ry12*$^{-/-}$ mice stained with antibodies directed against c-fos (green) and CGRP (red) on overlay image (merged). (c) The fluorescence intensity of CGRP-immunoreactive fibres is also significantly increased after NTG injection in wild-type mice (C1–C2: treatment effect: $F_{1,\ 20} = 16.159$, $P < 0.05$; TNC: treatment effect: $F_{1,\ 20} = 13.5859$, $P < 0.05$). Scale bar: 100 µm. Quantitative analysis of c-fos-IR cells (d) and CGRP immunoreactivity (e) in the C1–C2 and TNC after saline or PSB-0739 + NTG injection. (d) PSB-0739 significantly decreases c-fos-IR cells in the C1–C2 and TNC of wild-type mice (C1–C2: $P < 0.05$; TNC: $P < 0.05$). (e) PSB-0739 does not influence the density of CGRP-immunoreactive fibres (C1–C2: $P > 0.05$; TNC: $P < 0.05$; unpaired two-tailed Student's *t*-test). (f) Representative multi-immunofluorescence labelling images of C1–C2 and TNC from vehicle- and NTG-treated *P2ry12*$^{+/+}$ and *P2ry12*$^{-/-}$ mice stained with antibodies directed against c-fos (green) and CGRP (red) on overlay image (merged). Scale bar: 100 µm. Images were analysed and background subtracted in ImageJ. To measure fluorescent intensity, a region of interest was drawn and the average signal was measured. Values are presented as mean ± SEM; $^{*}P < 0.05$ compared with the vehicle-treated control group (two-way ANOVA with Tukey's post hoc test [a, c] and unpaired two-tailed Student's *t*-test [d])

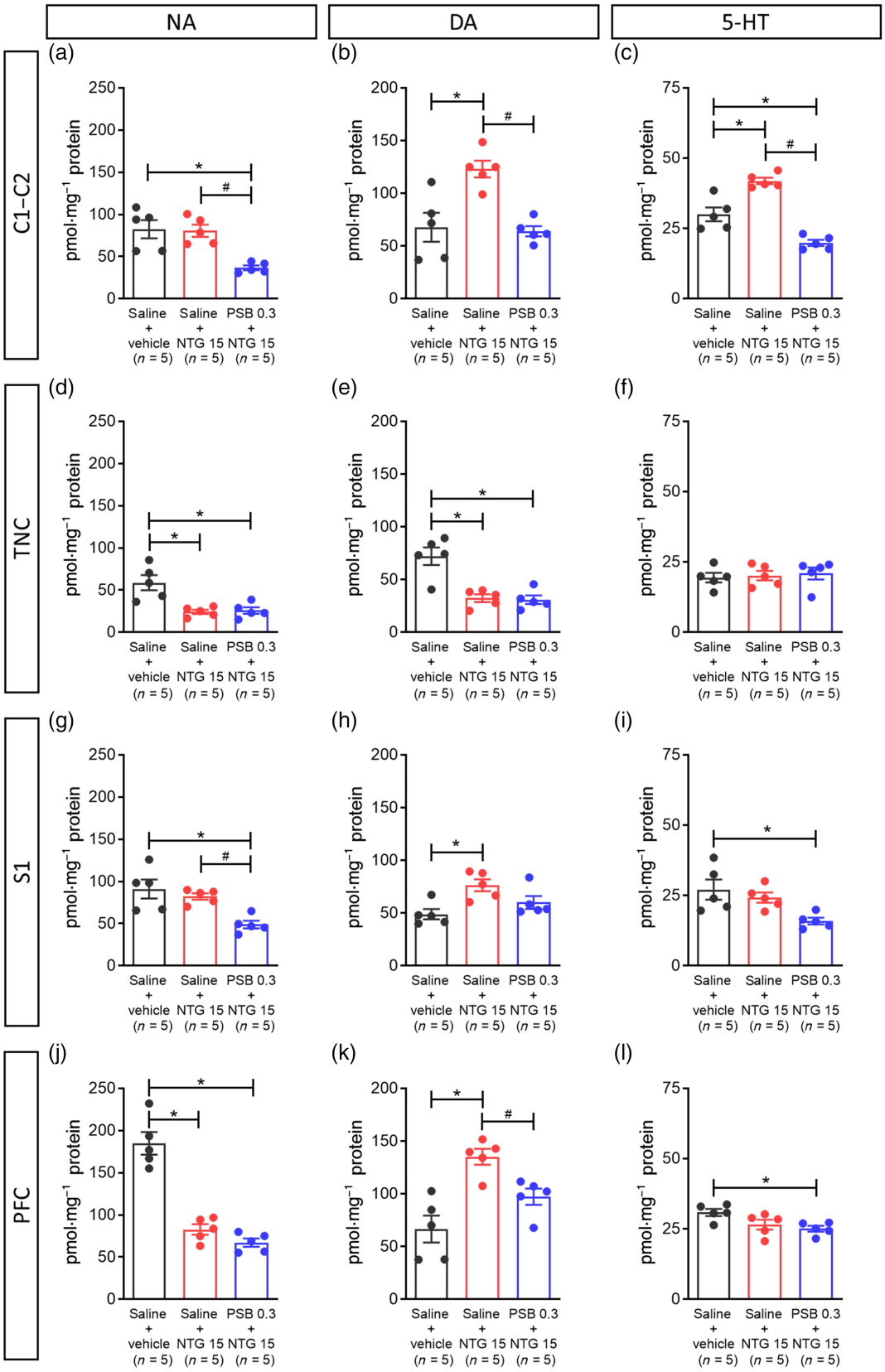

FIGURE 6 Neurotransmitter levels in *P2ry12*$^{+/+}$ mice after PSB-0739/saline and nitroglycerin (NTG) treatment. Monoamines were quantified using HPLC in the upper cervical spinal cord (C1–C2), trigeminal nucleus caudalis (TNC), the somatosensory cortex (S1) and prefrontal cortex (PFC) of mice. Concentrations of (a, d, g, j) noradrenaline (NA), (b, e, h, k) dopamine (DA) and (c, f, i, l) 5-hydroxytryptamine (5-HT; serotonin) were determined from samples 2 h after NTG administration. Values are presented as mean ± SEM, $^{*}P < 0.05$ compared with the saline + vehicle-treated group and $^{\#}P < 0.05$ compared with saline + NTG 15-treated group (one-way ANOVA with Tukey's post hoc test)

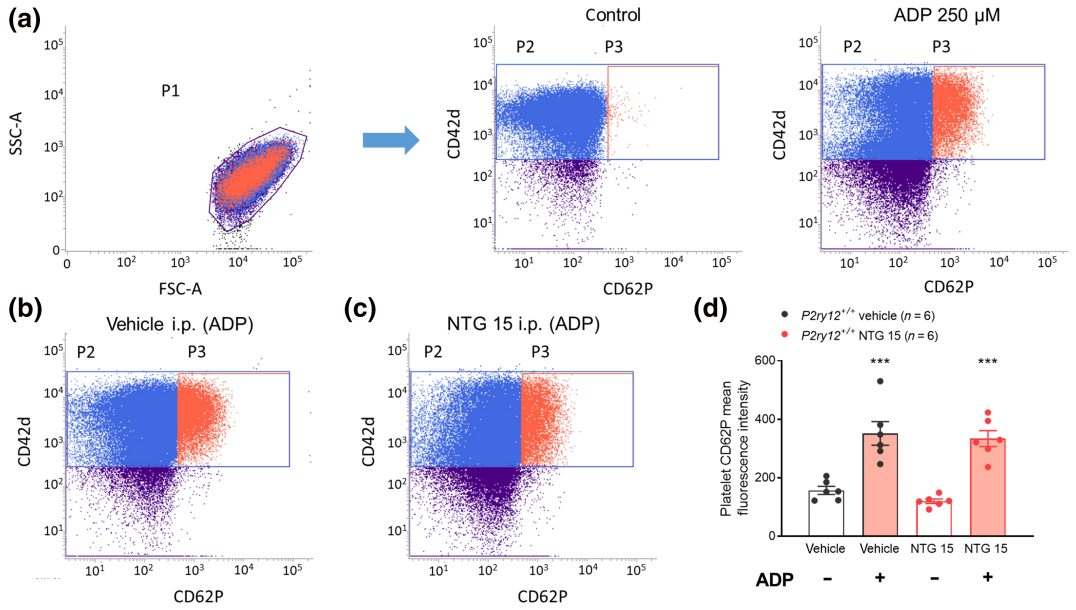

FIGURE 7 ADP-induced up-regulation of platelet CD62P is not affected by nitroglycerin (NTG) administration. (a) Representative flow cytometric dot plots showing ADP-induced platelet activation as identified by up-regulation of CD62P on CD42d^{+} platelets in *P2ry12*$^{+/+}$ mice. Platelet-rich plasma (PRP) samples were stimulated by 500-µM ADP. Platelets were gated on SSC/FSC dot plots (P1) to reveal ADP-activated platelets on CD42d/CD62P dot plots (P3). (d) Quantification of ADP-induced changes in platelet CD62P means fluorescence intensity values after ex vivo stimulation of PRP samples from intraperitoneally NTG-treated *P2ry12*$^{+/+}$ mice (induction effect: $F_{1,\ 33} = 61.0110$, $P < 0.05$; treatment effect: $F_{1,\ 33} = 1.1004$, $P > 0.05$). Data are expressed as a ratio of platelet CD62P fluorescence intensity in gate P2 and ADP-activated platelet CD62P fluorescence intensity in gate P3. Values are presented as mean ± SEM, $^{*}P < 0.05$ compared with control platelets (two-way ANOVA with Tukey's post hoc test). (b, c) Representative dot plots from each group after ADP stimulation ex vivo

4 | DISCUSSION

$P2Y_{12}$ receptor is well known as the platelet ADP receptor and the antithrombotic effect of its inhibition is widely used to prevent stroke and myocardial infarction. Recent investigations demonstrated that $P2Y_{12}$ receptors are also expressed by peripheral and central immune cells, for example megakaryocytes and microglia, regulating many aspects of inflammation and pain (Beko et al., 2017; Horvath et al., 2014; Yu et al., 2019). In the present study, we investigated $P2Y_{12}$ receptor involvement in the nitroglycerin-induced mouse model of migraine. To dissect endogenous $P2Y_{12}$ receptor-mediated actions, $P2Y_{12}$ receptor gene (*P2ry12*$^{-/-}$)-deficient mice and $P2Y_{12}$ antagonists were used. We also examined whether $P2Y_{12}$ receptors expressed by platelets play a role in nitroglycerin-induced hypersensitivity.

Nitroglycerin administration is known to induce a spontaneous-like migraine attack, which is associated with mechanical and thermal hypersensitivity in pain-free migraine patients and in mice (Demartini et al., 2019). Nitroglycerin treatment also causes other features of migraine, such as photophobia and up-regulation of c-fos and CGRP (Iyengar et al., 2017; Long et al., 2018; Markovics et al., 2012; Yi et al., 2018), as well as an alteration in monoamine levels (Wang et al., 2016; Tassorelli et al., 2002) in migraine-related brain areas in mice.

We also confirmed that acute nitroglycerin injection induced an initial decrease in BP in association with a transient increase in HR. By contrast, this effect failed to be observed at 1 h after nitroglycerin when behaviours were measured in wild-type and *P2ry12*$^{-/-}$ mice (Figure 1).

4.1 | Inhibition of centrally expressed $P2Y_{12}$ receptors attenuates nitroglycerin-induced migraine-like pain

Endogenous activation of $P2Y_{12}$ receptor is known to promote the development of neuropathic and inflammatory pain (Beko et al., 2017; Horvath et al., 2014; Yu et al., 2019). Our behavioural data showed that blocking $P2Y_{12}$ receptor activation by intrathecal administration of the selective antagonist, PSB-0739, attenuates nitroglycerin-induced thermal allodynia and head grooming in wild-type mice (Figures 2 and 3), whereas this effect was absent in $P2Y_{12}$ receptor gene-deficient mice, verifying the involvement of $P2Y_{12}$ receptors and implicating $P2Y_{12}$ receptor inhibition as a potential anti-migraine therapy. However, we could not see any differences between wild-type and $P2Y_{12}$ receptor gene-deficient mice in thermal hypersensitivity and head grooming after nitroglycerin treatment (Figures 2a and 3b). A possible explanation of this discrepancy could be that compensatory mechanisms in constitutively gene-deficient mice account for over-expression of other P2 receptors to mitigate the loss of a given gene. Similarly, in our earlier study, we showed that gene deficiency and pharmacological blockade evoked different behavioural responses in

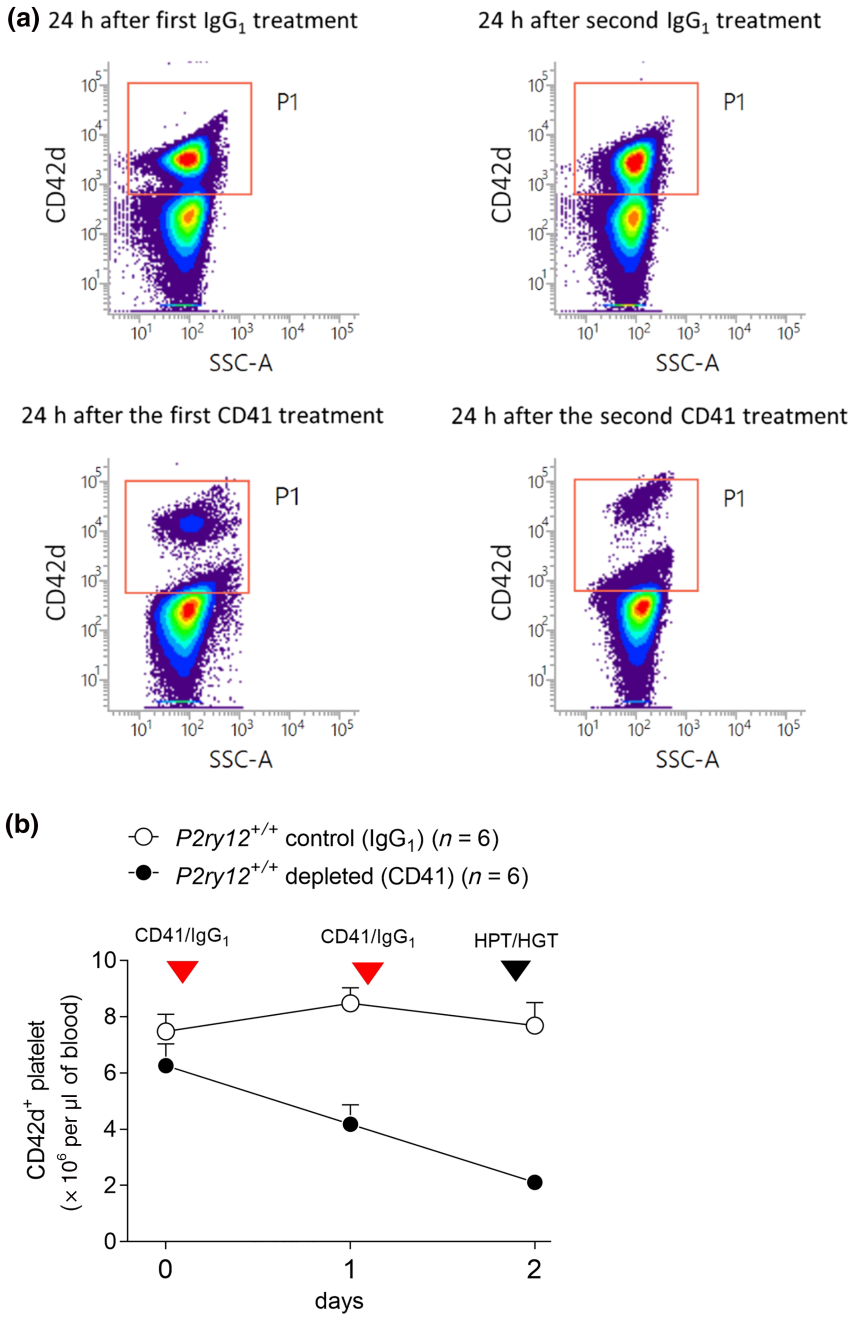

FIGURE 8 Validation of the efficiency of anti-mouse CD41-induced platelet depletion. Mice were subjected to intraperitoneal injections of anti-mouse CD41 antibody or IgG_1, κ isotype (control) antibody twice, before behavioural tests. (a) Dot plots show $CD42^+$ platelets (P1) in control and platelet-depleted mice. (b) The graph shows the duration of the effect of platelet depletion. Administration of CD41/IgG_1 (0.125 mg·ml^{-1}, i.p.) is indicated by red arrows and hotplate (HPT)/head grooming (HGT) tests are pointed by black arrow. Clopidogrel and its vehicle were administered on the second day after baseline paw withdrawal threshold (PWT) measurement and 30 min before NTG treatment in HPT. Values are presented as mean ± SEM

the case of another nucleotide receptor, **P2X7** (Goloncser & Sperlagh, 2014). Other studies have also revealed that the effect of $P2Y_{12}$ receptor gene deficiency and pharmacological blockade on the immune response are not identical (Ben Addi et al., 2010; Liverani et al., 2014). In our experiments, wild-type and $P2Y_{12}$ receptor gene-deficient mice did not show clear migraine-like photophobic behaviour in light-aversive test after nitroglycerin treatment. The results of light-aversion test obtained with nitroglycerin are still controversial (Vuralli et al., 2019). In our case, the lack of photophobia might be explained by different source or administration protocol of nitroglycerin, the time point when the behavioural tests were performed or the species of rodent.

Studies in animal models of neuropathic and inflammatory pain have shown that both mRNA and protein levels of $P2Y_{12}$ receptors are markedly increased in response to noxious stimuli (Gu et al., 2016; Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008; Yu et al., 2019). Although a significant up-regulation of $P2Y_{12}$ receptors has been postulated to occur in the TNC in wild-type mice in response to chronic nitroglycerin administration (Jing et al., 2019), we did not observe this phenomenon after acute nitroglycerin treatment (Figure 4). The lack of up-regulation in $P2Y_{12}$ receptor protein level coupled with the fact that $P2Y_{12}$ receptors are expressed mainly on microglia-like cells in the CNS, which are responsible for the maintenance of physiological neuronal activity and phagocytosis of damaged neuronal cells

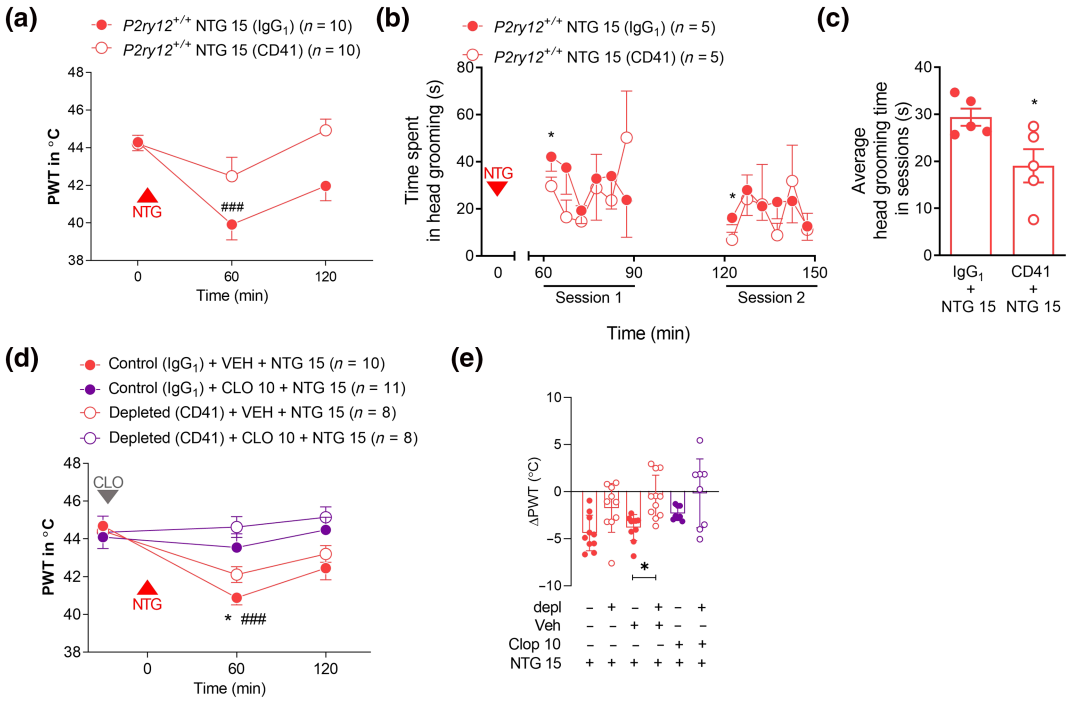

FIGURE 9 Effect of platelet depletion and $P2Y_{12}$ receptor inhibition on nitroglycerin (NTG)-induced thermal hypersensitivity and head grooming behaviour. Administration of NTG (15 mg·kg^{-1}, i.p.) is indicated by red arrows and clopidogrel and vehicle are pointed by grey arrow. CD42-treated mice show no difference in thermal sensitivity after dosing compared with the baseline measurement of IgG_1-treated control mice (see in Table S2). (a) Platelet depletion suppresses thermal hyperalgesia (time × treatment effect: $F_{2, 36} = 4.11$, $P< 0.05$), clopidogrel (10 mg·kg^{-1}) is also effective (time × pretreatment effect: $F_{2, 36} = 9.60$, $P< 0.05$) and their effect is additive (d) (depletion × pretreatment effect: $F_{1, 33} = 0.00$, $P > 0.05$). (e) Platelet depletion and clopidogrel reverse thermal hypersensitivity 1 h after NTG treatment (depletion effect: $F_{1, 51} = 6.2904$, $P< 0.05$; pretreatment effect: $F_{2, 51} = 8.8299$, $P< 0.05$; and depletion × pretreatment effect: $F_{2, 51} = 1.2252$, $P >0.05$). (b, c) Platelet depletion is effective in alleviating NTG-induced head grooming in *P2ry12*$^{+/+}$ mice ($P< 0.05$). The time spent in head grooming was recorded in the two 30-min test session and divided to 5-min intervals. (e) Changes in paw withdrawal threshold (PWT) of depleted and non-depleted mice after treatments at 1 h were calculated by ΔPWT = PWT at 1 h − baseline PWT in °C formula. Values are presented as means ± SEM; $^{*}P < 0.05$ compared with control group (vehicle treated/non-depleted) at the same time point and $^{\#}P < 0.05$ compared with baseline PWT values of the same treatment group (repeated measures ANOVA with Tukey's post hoc test [a, d]; Friedman test with Wilcoxon matched-pairs test [b]; unpaired two-tailed Student's *t*-test [c]; and two-way ANOVA with Tukey's post hoc test [e])

(Cserep et al., 2020; Fekete et al., 2018), indicate that neuroinflammation has a limited influence on the observed $P2Y_{12}$ receptor-dependent changes during the acutely induced mouse model of migraine.

CGRP, a pivotal neuropeptide in the trigeminal system involved in both peripheral and central sensitisation (Cottrell, 2019; Iyengar et al., 2017), has been implicated as one of the major pathophysiological mechanisms underlying migraine. Similarly, the involvement of c-fos, an immediate early gene widely used as a general neuronal activity marker (Kovacs, 2008), has also been shown in central sensitisation. We have observed increased levels of CGRP and c-fos in spinal C1–C2 and TNC in wild-type mice in association with hypersensitivity in our migraine model; the latter was suppressed by PSB-0739 administration (Figure 5). Because c-fos is produced by neurons, the inhibitory effect of $P2Y_{12}$ blockade on c-fos may be due to involvement in microglial–neuronal interaction (Cserep et al., 2020). Consistent with the findings on thermal hypersensitivity and head grooming behaviour, the c-fos level was significantly increased in both wild-type and $P2Y_{12}$ receptor gene-deficient mice after nitroglycerin treatment. The increase of nitroglycerin-induced c-fos expression in gene-deficient mice can be explained by the modulatory effect of other receptors (e.g. **5-HT$_{1B}$**, 5-HT$_{1D}$, **5-HT$_{1F}$**, **GABA$_A$**, **NMDA** and **opioid μ**) in TNC and related nociceptive pathways. Interestingly, nitroglycerin treatment did not affect CGRP level in gene-deficient mice. One possible explanation for this observation is that $P2Y_{12}$ receptor signalling deficiency results in a lack of **p38 MAPK** activation in spinal microglia (Kobayashi et al., 2008), which can modulate CGRP synthesis and release (Durham, 2006). **NGF** and **BDNF** are also potential factors influencing CGRP synthesis, release and activity (Salio et al., 2007; Supowit et al., 2001), which can be modulated by **P2X3 receptor** (Ceruti et al., 2011) and microglial **P2X4** receptor via PGE_2 (Long et al., 2020), whereas the expression of both genes may also be elevated in $P2Y_{12}$ receptor gene-deficient mice. Previous research found that MRS2395, a $P2Y_{12}$ antagonist, reduced CGRP expression in the trigeminal ganglion and TNC using another chronic nitroglycerin-induced protocol of migraine model (Jing et al., 2019; Sugawara et al., 2017). In contrast, in our study PSB-0739 did not affect CGRP expression in either the spinal cord or TNC. Here, we have to note that, however, MRS2395 is not a selective $P2Y_{12}$ receptor antagonist,

having effects unrelated to $P2Y_{12}$ inhibition (Horvath et al., 2014). In that study, it was also shown that experimental vagotomy prevented the effect of intrathecal PSB-0739 treatment on complete Freund's adjuvant (CFA)-induced mechanical hyperalgesia measured in the periphery, suggesting that a similar mechanism may also play a role in our experimental conditions. Nevertheless, the central effect of PSB-0739 on neuronal activation, reflected in c-fos activation, is the most likely primary underlying mechanism of attenuating nitroglycerin-induced migraine-like pain in our experiments.

Microglial cells can produce and release a variety of soluble mediators including both pro- and anti-inflammatory mediators, cytokines, chemokines (Turner et al., 2014) and growth factors being key modulators of acute and chronic inflammatory processes. P2X and P2Y receptors are known to affect the processing and secretion of many soluble microglial mediators (Calovi et al., 2019).

In this study, we found that acute nitroglycerin injection did not cause any change in cytokine level in serum in wild-type mice (Figure S3) nor did PSB-0739 administration (Figure S4). This finding implicates that acute nitroglycerin treatment does not evoke a robust systemic inflammatory response, at least not at the time point when the analysis was performed.

We also observed that nitroglycerin administration modulated the level of monoamine transmitters in various CNS regions relevant to migraine, such as the C1–C2, TNC, the somatosensory cortex (S1) and prefrontal cortex, which were partly controlled by central blockade of $P2Y_{12}$ receptor by PSB-0739 (Figure 6) or by the primarily peripherally acting drug clopidogrel (Figure S5). Descending axons from noradrenergic locus coeruleus in the pons project to pain pathway circuitry at the spinal dorsal horn and trigeminal nerve entry zone in TNC (Agster et al., 2013; Millan, 2002; Panneton et al., 2011). Thus, noradrenaline may play an essential role in primary headache disorder (Benarroch, 2018; Bussone, 2008). 5-HT has been widely associated with pain modulation through peripheral and central actions. In contrast to noradrenaline, whose actions seems to be more related to analgesia, 5-HT triggers specific receptors contributing to pain maintenance (Bannister & Dickenson, 2016; Suzuki et al., 2004). In our study, we found that noradrenaline level decreased in TNC and prefrontal cortex but was unchanged in C1–C2 and S1 after nitroglycerin treatment, whereas the level of 5-HT was increased only in C1–C2 after nitroglycerin administration. Our results are consistent with previous findings, where noradrenaline and 5-HT levels exhibited no significant difference in the pons and medulla at 2 h after nitroglycerin injection in rats (Tassorelli et al., 2002). Additionally, a recent study also shows that the levels of noradrenaline and 5-HT were not changed after complete Freund's adjuvant injection in TNC and S1 in a complete Freund's adjuvant-induced orofacial pain rat model (Cseh et al., 2020). A possible explanation for the discrepancies, having found a marked decrease in noradrenaline levels in the TNC area, could be the difference in the experimental settings and rodent species. It was recently reported that descending dopaminergic pathways contribute to pain transmission (Dias et al., 2015; Dieb et al., 2016; Kim et al., 2015), and it may depend on local dopamine concentration, which activates various dopamine receptors, so it can inhibit or enhance pain (Liu et al., 2019). We found significant changes in dopamine level in all four areas after nitroglycerin injection. Further research is required to identify possible mechanisms by which neurotransmitters interact with primary headache diseases. The purinergic modulation of the pain transmission has been described (Burnstock, 2013) and numerous studies reported the interaction between purinergic and monoaminergic pathways (Arribas-Blazquez et al., 2019; Horvath et al., 2014; Peroutka, 2004; Suleimanova et al., 2020). ADP-sensitive receptors and monoamine receptors are also expressed both in trigeminal neurons and glial cells of the trigeminal system (Villa et al., 2010). We have shown that PSB-0739 significantly decreased noradrenaline, dopamine and 5-HT levels in C1–C2, noradrenaline level in S1 and dopamine level in prefrontal cortex. These findings suggest that $P2Y_{12}$ receptor in the spinal cord may play an important role in the initiation and maintenance of migraine-like pain via the monoamine system. Further studies are needed to better define the functions of purinergic receptors in pain modulation.

Our results demonstrate that intrathecally administered PSB-0739 has a uniform distribution throughout the neuraxis and is able to reach the cervical spinal cord and even the brain (Figure S1). Nonetheless, we cannot entirely exclude the possibility that the compound itself can get to the periphery from the CNS, but the extent of this appears negligible.

4.2 | Peripheral inhibition of $P2Y_{12}$ receptor on platelets mitigates nitroglycerin-induced migraine-like pain

Several studies have explored potential associations between platelet biology and migraine. Spontaneous platelet activation and aggregation were observed during and between migraine attacks (Danese et al., 2014). Among extracellular nucleotides, ADP plays a central role in platelet function, although it is a weak platelet agonist only inducing reversible responses including aggregation. Platelets express **$P2Y_1$** and $P2Y_{12}$ receptors. $P2Y_{12}$ receptor plays an important role in the activation and the stabilisation of platelet aggregates via PI3K and **Akt** activation and inhibition of adenylate cyclase (Cattaneo, 2019; Hechler & Gachet, 2017). In the present study, we showed that nitroglycerin treatment per se did not affect ADP-induced up-regulation of platelet CD62P 2 h after its administration (Figure 7). Therefore, our result is consistent with previous findings that the inhibitory effect of nitroglycerin on platelet function and ADP-induced aggregation is rapidly reversible after discontinuation of the drug (Aoki et al., 1997).

On the other hand, we show that platelet depletion decreases hyperalgesia and head grooming time evoked by nitroglycerin (Figure 9), pointing to the role of ADP and other mediators released from platelets in migraine-like pain. We also show that noradrealine, dopamine and 5-HT levels decrease in C1–C2 and S1, but remained unchanged in the TNC and prefrontal cortex (see in Figure S5). Upon activation, platelets secrete various mediators, such as cytokines, chemokines, 5-HT and NO (Danese et al., 2014; Slaba & Kubes, 2017; Turner et al., 2014). The release of these mediators plays an important role in paracrine signalling

of platelets, for instance, immune cell activation, proliferation and chemotaxis (Turner et al., 2014), whereas during platelet depletion, this modulating effect is entirely lost. This may also result in disturbed signalling in the CNS (Dukhinova et al., 2018). In the periphery, $P2Y_{12}$ receptor is highly expressed on platelets, where the receptor appears to be involved in the expression of various inflammatory factors such as IL-1β, IL-6 and TNF-α: inhibition of the receptor with clopidogrel reduced pro-inflammatory mediator levels in the plasma, notably IL-1β, IL-6 and TNF-α (Hechler & Gachet, 2017). Clopidogrel was also associated with enhanced phosphorylation of Akt and endothelial NOS (Nylander & Schulz, 2016). We show that clopidogrel treatment decreased noradrealine, dopamine and 5-HT levels in C1–C2, to the same extent as observed after platelet depletion (Figure S5). Furthermore, clopidogrel and platelet depletion had an additive effect on nitroglycerin-induced hyperalgesia and changes of monoamine levels in CNS, strongly suggesting that the platelet-mediated effect is the result of a complex interplay between platelet-derived mediators and also involves receptors other than $P2Y_{12}$ receptors.

In the present study, we used exclusively male mice due to the possibility that female mice treatment responses might be affected by the phase of oestrous cycle and sex steroid hormones (Bolay et al., 2011; Ebine et al., 2016). However, it would be worthwhile to study the involvement of $P2Y_{12}$ receptor in migraine models using female mice in the future as women suffer from migraine three times as often as men (Charles, 2017; Dodick, 2018).

5 | CONCLUSION

In conclusion, we demonstrated that selective inhibition of central $P2Y_{12}$ receptor alleviates acute nitroglycerin-induced hyperalgesia and modulates neuronal activation in the TNC and C1–C2. Inhibition of platelet $P2Y_{12}$ receptor might contribute to these effects. This study provides new insight into the participation of $P2Y_{12}$ in migraine and supports its role as a potential therapeutic target for headache disorders.

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AUTHOR CONTRIBUTIONS

F.G. carried out the behaviour, IHC and FACS experiments, analysed the data, performed the statistical analyses and drafted the manuscript. M.B. conducted the HPLC analysis. A.I. and L.H. performed the BP and heart rate measurement and analysed the data. L.O. carried out confocal microscopy and revised the manuscript. Z.B. designed the experiments and revised the manuscript. B.S. designed and supervised the study and finalised the paper. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

ORCID

Beáta Sperlágh https://orcid.org/0000-0003-0099-591X

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