FINAL REPORT

on the research grant of the National Research Development and Innovation Office, entitled

„THE ROLE OF P2Y12 RECEPTORS IN CONTROL OF PAIN AND NEUROINFLAMMATION"

Grant No: 116654

Introduction and objectives

P2Y12 receptors (P2Y12Rs) are metabotropic receptors activated primarily by endogenous ADP. They belong to rhodopsin subfamily of G-protein coupled receptors (GPCR), and consist of a single polypeptide chain of 342 amino acids, with 7 transmembrane regions, 3 extracellular (EL1, EL2, EL3) and 3 intracellular loops (Figure 1). P2Y12Rs are expressed predominantly on platelets, but also on other cell types, such as the cells of the central nervous system, including microglia and nerve terminals. The activation of P2Y12Rs by ADP leads to the recruitment of Gi/α proteins and the inhibition of cAMP production and this effect leads to the rapid aggregation of platelets. In addition, various other actions have been described to be mediated by P2Y12Rs, such as the activation of microglia following brain insults (Haynes et al., 2006) and the regulation of neurotransmitter release during ongoing neuronal activity (Boehm, 1999, Heinrich et al., 2008). Previous investigations by our group and others showed that genetic deletion and pharmacological inhibition P2Y12R leads to the alleviation of neuropathic and acute inflammatory pain (Andó et al., 2010; Horvath et al., 2014)

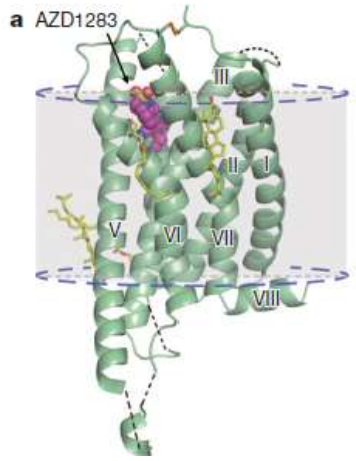

Figure 1. The structure of P2Y12R in antagonist (AZD1283) bound state. From Zhang et al. (2014).

Therefore in this project our general aim was to extend these observation to chronic conditions and the precise understanding of the actions mediated by $P2Y_{12}$Rs in different animal models of neuroinflammatory disorders. Among potential pathophysiological states accompanied by pain, we concentrated on chronic inflammatory pain and migraine. In addition, because the expression of $P2Y_{12}$Rs are concentrated in the central nervous system to amygdala, hippocampus, caudate nucleus, substantia nigra, corpus callosum and thalamus (Hollopeter et al., 2001) we have focused to those diseases, which affect the above brain areas and which are characterized by accompanying neuroinflammation: Parkinson's disease and depression.

The original specific aims were the following:

1. To establish the role of $P2Y_{12}R$ in chronic CFA induced hyperalgesia and local inflammation and to identify the different cell types and signaling pathways mediating these effects
2. To explore, whether genetic deletion and pharmacological inhibition of $P2Y_{12}Rs$ is protective in a mouse model of migraine
3. To explore, whether genetic deletion and pharmacological inhibition of $P2Y_{12}Rs$ is protective in a mouse model of diabetic neuropathy
4. To examine the potential involvement of $P2Y_{12}$ receptors in animal models of Parkinson's disease (PD) and other neuroinflammatory disorders

Results

1. Identification of the role of P2Y12Rs in hyperalgesia and inflammation in chronic CFA induced inflammatory pain

The research was performed according to the working plan and we have investigated the role of P2Y12 receptors in chronic inflammatory pain in mice and explored the contribution of platelet P2Y12 receptors to the observed effects.

Complete Freund's adjuvant (CFA)-induced chronic inflammatory pain was induced in wild-type and P2ry12 gene-deficient mice (P2ry12-/-), and the effects of the potent, selective P2Y12 receptor antagonist, PSB-0739 and the direct-acting and reversible P2Y12 receptor antagonist cangrelor were examined. Wild-type and P2ry12-/- mice were subjected to an intraplantar CFA injection and the paw withdrawal threshold (PWT) was determined on the 3^{rd} , 4^{th} , 7^{th} , 10^{th} and 14^{th} days. Three days after the intraplantar CFA injection, the PWT values were significantly decreased in P2ry12+/+ mice, indicating the development of mechanical hyperalgesia. This was accompanied by an increase in paw volume, the inflammatory edema. Both hyperalgesia and edema were maintained up to 14 days. A robust increase in neutrophil MPO activity was also observed in wild-type mice on the 3^{rd} day, which was attenuated but remained detectable at the 10^{th} day. In addition, CFA-treatment elicited an increase in pro-inflammatory cytokine levels in hind paws. IL-1β, IL-6, TNF-α and KC levels increased further by 14 days compared to that measured 3 days after CFA-treatment in the P2ry12+/+ mice.

In P2ry12-/- mice, mechanical hyperalgesia was significantly decreased compared with wild-type mice and the difference between the genotypes was maintained 14 days after the CFA injection. Likewise, the CFA-induced increase in neutrophil MPO activity was significantly less intensive in the P2ry12-/- mice on the 3^{rd} day compared to the wild-type mice; however, no genotype-related difference was detected on the 10^{th} day. Paw edema was not affected by the genotype at any of the examined time points, therefore, paw swelling was subsequently not assessed. Genetic deletion of P2Y12Rs counteracted the pro-inflammatory cytokine response at both time points: on the 3^{rd} day, the TNF-α and KC level inductions were significantly attenuated, and a tendency for decreased IL-6 levels was also observed compared with the wild-type mice. IL-1α remained undetectable in P2ry12-/- mice but not in wild-type mice. The CFA-induced elevation in the IL-1β, IL-6, TNF-α and KC levels was significantly alleviated in the P2ry12-/-animals on the 14^{th} day compared with the wild-type mice. CFA downregulated the anti-inflammatory cytokine IL-10 in the wild-type mice and this effect was not altered by the genotype on the 3^{rd} day.

Next, we attempted to reproduce the anti-hyperalgesic effect of P2Y12R deficiency with specific antagonists. At first, we used PSB-0739, a potent and selective antagonist of P2Y12Rs having nanomolar potency at hP2Y12 (pKB=10.1 in cAMP assay, pA2=9.8 in reporter gene assay) and without affinity of any other P2 receptor below 1 μM. Because PSB-0739 does not

penetrate the blood brain barrier (BBB), animals were treated intrathecally. In wild-type mice, PSB-0739 (0.3 mg/kg i.t.) had a significant anti-hyperalgesic effect compared with the saline-treated control group at every time points from the 4th day up to the 14th day. In contrast, hyperalgesia was not changed by PSB-0739 in the P2ry12-/- group compared with the saline-treated control group.

In another set of experiments, animals were treated with intraperitoneal injection of the direct acting, reversible P2Y12R antagonist, cangrelor (pKB=8.6-9.2 for hP2Y12 receptor). Cangrelor has no significant activity at other P2 receptors in concentrations below 3 μM besides the human P2Y13, which binds it with nanomolar affinity. Cangrelor alleviated mechanical hyperalgesia in wild-type mice similar to PSB-0739. However, cangrelor elicited a slight, but significant pro-hyperalgesic effect in the P2ry12-/- mice compared to the saline-treated control group.

To compare the anti-hyperalgesic effect of P2Y12R antagonists with an alternative platelet antagonist, the effect of low dose aspirin (20 mg/kg i.p.) was also evaluated. In contrast to PSB-0739 and cangrelor, aspirin had no effect in the early phase (4th day), but a gradual anti-hyperalgesic effect developed in the later phase of inflammation, which became similar to the effect of cangrelor by the 14th day. This mild anti-hyperalgesic effect of aspirin was also detected in P2ry12-/- mice.

Next, we examined whether the local P2Y12R blockade has any effect on CFA-induced hyperalgesia. Interestingly, PSB-0739 (0.3 mg/kg i.pl.), when administered to the plantar surface of the inflamed hind paw had significant anti-hyperalgesic effect compared with the saline-treated control group. To identify the underlying neuronal signaling mechanism involved, we examined whether this anti-hyperalgesic effect could be attenuated by blocking axonal conduction in sensory nerves. To this end, A-803467 a selective and potent antagonist of NaV1.8 channels was used (IC_{50}=8nM for hNaV1.8), showing no significant activity against TRPV1, P2X2/3, CaV2.2 and KCNQ2/3 channels. Consistently with literature data, A-803467 (30 mg/kg i.p.) relieved hyperalgesia, when compared with vehicle treatment, although complete reversal was not obtained at this dose. However, no further anti-hyperalgesic effect was obtained by either intrathecal or intraplantar PSB-0739 treatment after pre-treatment with A-803467.

Because the majority of peripheral P2Y12Rs are expressed on platelets, we examined whether P2Y12-driven actions in CFA-induced hyperalgesia require the presence of functional platelets. To this end, mice were treated with anti-mouse CD41 antibody (25 μg i.p.) 6 days after CFA administration, which resulted in an almost complete depletion of platelets from day 7 (11.75%) up to 14 days (8.54%). Leukocyte populations were not affected by the antibody-treatment. On the 7th and 10th days, platelet deficiency did not influence mechanical sensitivity; however, on the 14th day (when the inflammatory cytokine responses peaked), platelet depletion reduced hyperalgesia in wild-type mice, but not in P2ry12-/- mice compared with the control (anti-IgG1-treated) groups. As shown above, P2Y12R deficiency by itself significantly alleviated hyperalgesia at all time points in the control groups.

When the cytokine profile was analyzed in hind paw samples collected on the 14th day, both platelet depletion and genotype influenced the pro-inflammatory cytokine response. Similar to aesthesiometry findings, platelet depletion had a significant anti-inflammatory effect in the wild-type but not in the P2ry12-/- mice, as shown by a significant decrease in IL-1β, IL-6, TNF-α and KC levels in the anti-CD41-treated animals compared to the anti-IgG1-treated mice. Moreover, platelet depletion slightly increased IL-6 and KC levels in P2ry12-/- animals compared to the wild-type mice. In contrast, IL-10 levels did not significantly change in platelet depleted animals of either genotype, when compared their control. As observed earlier in the

absence of P2Y12R, the IL-1β, IL-6, TNF-α and KC levels were lower compared to their wild-type littermates.

Finally, we examined how identical treatments with P2Y12R antagonists and the reference compound aspirin influence platelet activation, by assessing ADP-induced changes in platelet CD62P levels ex vivo in platelet rich plasma (PRP) samples. ADP (500 μM) induced a significant upregulation (296.47±22.14% and 309.21±12.99% in the i.p. and i.t. saline-treated groups, respectively, n=8, $P<0.01$) of CD62P on CD42d-positive platelets. Cangrelor (3 mg/kg i.p.) almost completely reversed this effect. Aspirin (20 mg/kg i.p.) and PSB-0739 (0.3 mg/kg i.t.) had no effect on ADP-induced platelet activation, when compared to their respective controls.

In conclusion, P2Y12 receptors regulate CFA-induced chronic hyperalgesia and local inflammatory response, and platelet P2Y12 receptors contribute to these effects in the chronic inflammation phase. These results are published (**Bekő K, Koványi B, Gölöncsér F, Horváth G**, Dénes Á, Környei Z, Botz B, Helyes Z, Müller CE, **Sperlágh B**. (2017) Contribution of platelet P2Y12 receptors to chronic Complete Freund's adjuvant-induced inflammatory pain. J Thromb Haemost. 15(6):1223-1235.).

2. Identification of the role of P2Y12 receptors (P2Y12Rs) in a nitroglycerin (NTG) induced mouse model of migraine

The involvement of P2Y12Rs in chronic pain demonstrated in our previous studies (Horvath et al., 2014; Bekő et al., 2017) implies its role in other pain modalities, such as migraine. Other studies suggest that P2Y12 receptors are upregulated in satellite glia cells of trigeminal ganglion (Ceruti et al., 2008, Katagiri et al., 2012) during chronic pain and might modify the nociceptive information processing through the trigeminal nerve. Therefore, it was worthwhile to examine how genetic deletion and pharmacological antagonism of P2Y12Rs affect hyperalgesia in a widely used migraine model. We followed the procedures validated and applied by our group in an earlier study (Goloncser and Sperlagh, 2014). Migraine-like pain and behavioral alterations was induced by a single intraperitoneal NTG injection in wild-type and P2ry12-/- mice using an increasing-temperature hot plate system, head grooming and light/dark box tests. NTG induced thermal hyperalgesia, increased head grooming time and photophobia of wild-type mice, followed by the induction of c-fos and CGRP in upper cervical spinal cord (C1-C2) and trigeminal nucleus caudalis (TNC). These changes also were observed in P2ry12-/- mice. The selective $P2Y_{12}R$ antagonist PSB0739 (0.3 mg/kg i.t.) or its vehicle (saline) was administered 15 min before NTG, or applied as a post-treatment (Figure 2). Prophylactic application of PSB-0739 reversed thermal hyperalgesia and head grooming time in wild-type mice but had no effect in P2ry12-/- mice, and it was also effective when applied as a post-treatment. PSB-0739 also suppressed the expression of c-fos and CGRP in C1-C2 and TNC in wild-type mice (Figure 3).

Cytokines are possible biomarkers of the inflammatory response during migraine attacks, and the changes in the level of neurotransmitters in migraine related areas of the CNS might also be indicators of the alterations in central nociceptive information processing. Flow cytometry analyses and HPLC were used to evaluate the level of inflammatory cytokines in serum and endogenous neurotransmitters in brain areas, respectively. Two hours after acute NTG treatment, we did not find any changes in cytokine level in the serum and the majority of monoamine levels in C1-C2, TNC, somatosensory cortex (S1) and prefrontal cortex (PFC) in wild type mice, whilst prophylactic PSB-0739 significantly decreased the level of IL-1β and IL-6. The level of serotonin (5-HT) was significantly increased in S1 and PFC in wild-type mice, while its level was decreased in PFC in P2ry12-/- mice after NTG treatment. Wild-type

mice that received PSB-0739 showed significantly decreased levels of noradrenaline, dopamine and 5-HT in C1-C2 and S1 regions.

Platelet depletion was used to investigate the role of platelet P2Y12Rs in the course of migraine-like pain. NTG treatment itself did not change ADP-induced platelet activation measured by CD62P upregulation in wild-type mice. Platelet depletion by anti-mouse CD41 antibody attenuated NTG-induced thermal hypersensitivity. Clopidogrel (10 mg/kg i.p.) is a non-selective P2ry12 antagonist, similar to the effect of platelet depletion, also significantly attenuated NTG induced hyperalgesia. When we applied platelet depletion and clopidogrel treatment together the NTG induced hyperalgesia is almost completely abolished (Figure 4). These data indicate that endogenous P2Y12R activation and other substances released from platelets contribute to the development of migraine-like hypersensitivity.

In conclusion, we found that whereas P2ry12 gene deficiency had no effect, acute pharmacological blockade of P2Y12 effectively alleviated NTG-induced hyperalgesia and related neurobiological parameters (c-fos, CGRP) in the migraine-related brain areas.

The manuscript prepared from these results is ready for submission (**F. Gölöncsér, M. Baranyi, L. Otrokocsi, B. Sperlágh**, Involvement of P2Y12 receptors in an NTG-induced mouse model of migraine to be submitted to British Journal of Pharmacology).

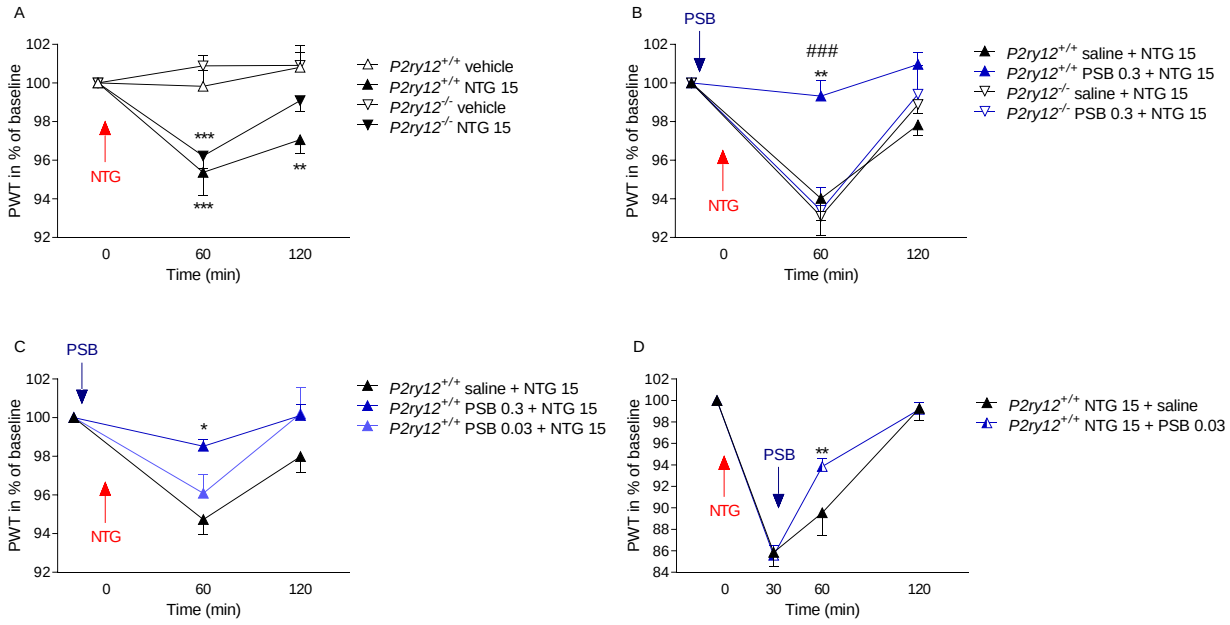

Figure 2. **NTG induces thermal hypersensitivity in mice which reverses with the P2Y$_{12}$ antagonist PSB-0739 in *P2ry12*$^{+/+}$, but not in *P2ry12*$^{-/-}$ mice.** Data show mean ±SEM. N=5-32/group.

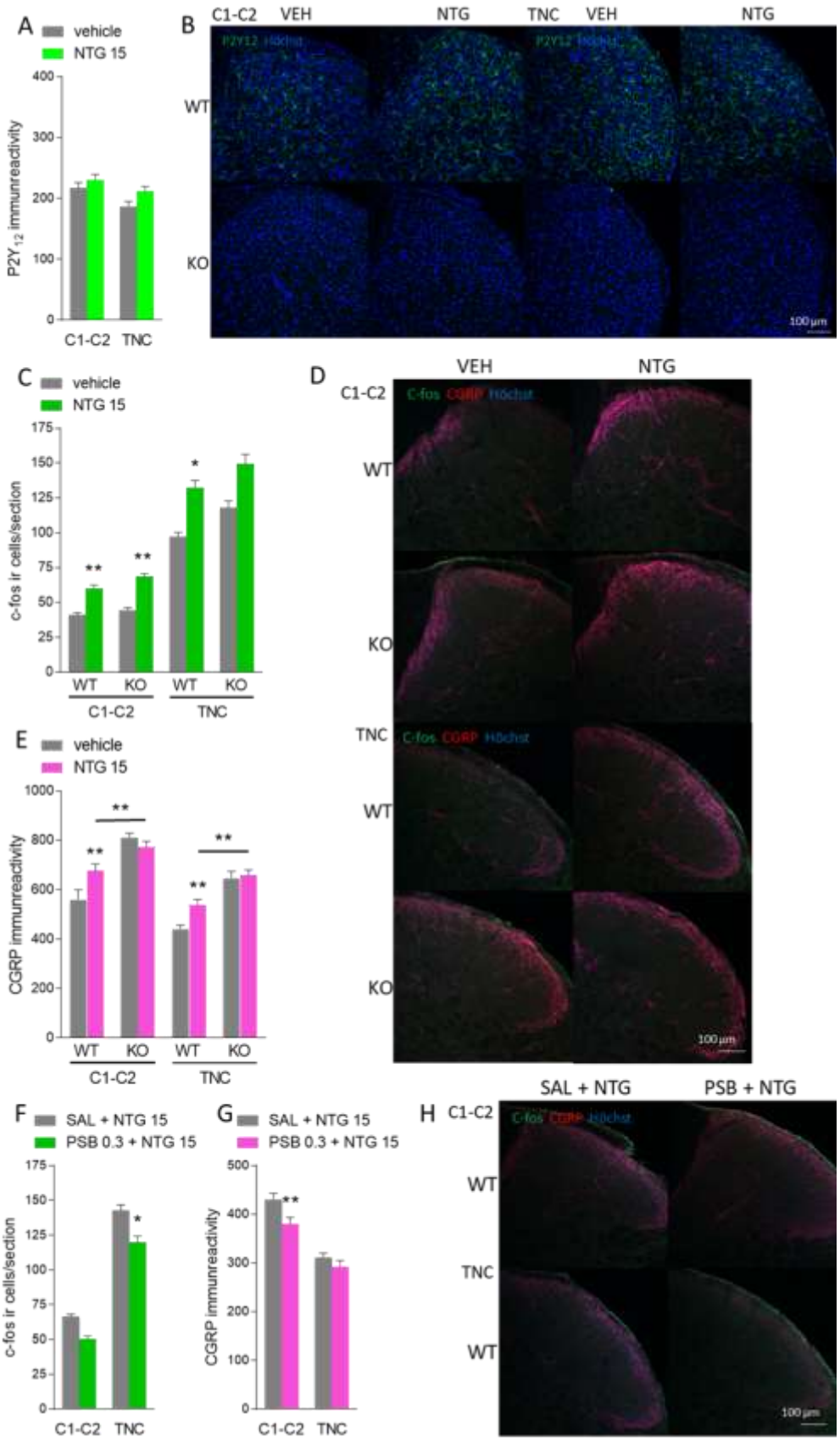
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Figure 3. **Effect of NTG administration on P2Y$_{12}$R, c-Fos and CGRP expression in wild-type and *P2ry12-/-* mice.** Inhibition of P2Y$_{12}$R function reduced NTG-induced c-fos and CGRP expression in TNC. Data show mean ±SEM. N=4-5 mice/group.

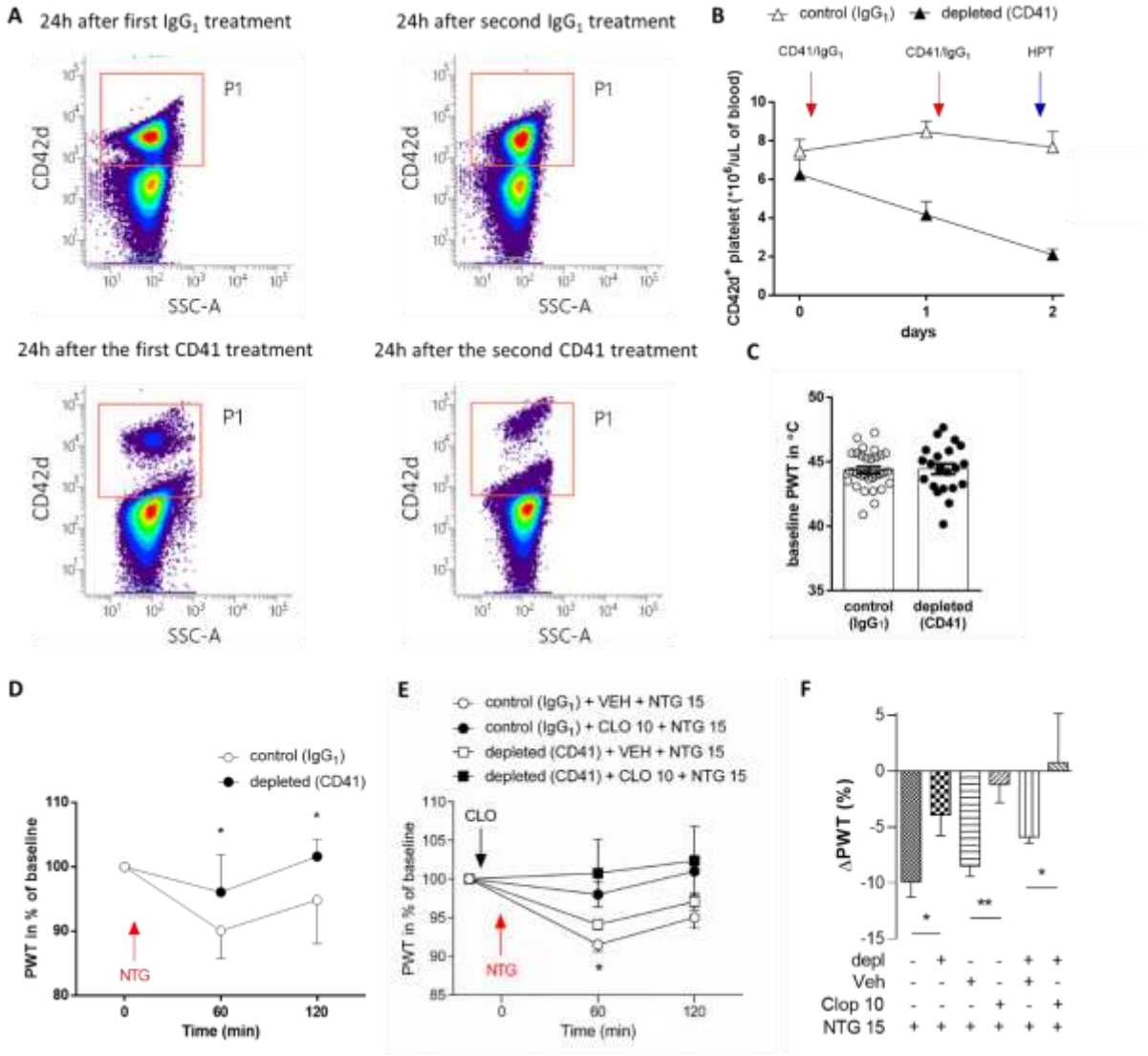

Figure 4**. Platelet depletion and P2Y$_{12}$ antagonist attenuated NTG-induced thermal hypersensitivity in wild-type mice.** Mice were subjected to i.p. injections of anti-mouse CD41 antibody for deplete platelet or IgG1, κ isotype (control) antibody twice, before NTG-induced hot plate test. Data show mean ±SEM. N=5-11 mice/group.

3. The potential role of P2Y$_{12}$Rs in a mouse model of diabetic neuropathy

Because in the meantime, another group has published data on the role of P2Y12Rs in diabetic neuropathic pain (Wang et al., 2018), we have decided to omit this objective. Instead, we have participated in collaborations revealing further important and uncovered aspects of P2Y12R function in neuroinflammation and brain pathophysiology. The results are detailed in **6-8**.

4. The potential involvement of P2Y12 receptors in animal models of Parkinson's disease (PD)

Neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease (PD), are often coupled to neuroinflammation. Recent investigations revealed that P2Y12 receptors (P2Y12R) are important mediators for the initiation of neuroinflammation by regulating microglia activation and function; however, the effect of P2Y12R deficiency on the neurodegenerative Parkinson's disease has not been explored yet. In the present study, we have investigated the effect of P2Y12R deficiency, either via genetic deletion or pharmacological inhibition, using the acute MPTP induced PD model, as described in our previous experiments (Hracsko et al., 2011, Baranyi et al., 2016). Adult male wild type and p2ry12-/- mice were used in all experiments and animals were injected (i.p.) with sterile saline (0.9% NaCl) or 4 x 20mg/kg MPTP, separated by 2 h. Mice were euthanized by decapitation 72 h after the last treatment. The striatum and the substantia nigra pars compacta (SN) were collected, frozen on dry ice and stored at −70°C until homogenization. The tissue content of endogenous dopamine and its metabolites (DOPAC, HVA) as well as other transmitters and modulators (noradrenaline, 5-HT, GABA glutamate, nucleotides, endocannabinoids) were analyzed by HPLC. Tyrosine hydroxylase (TH) immunohistochemistry was performed on striatal and SN sections of MPTP or saline treated animals. The open field and rotarod tests, 2 and 24 hours after the MPTP / saline treatments, respectively, monitored motor function.

Administration of MPTP induced selective death of dopaminergic neurons in the substantia nigra and a profound depletion of the content of endogenous dopamine and its metabolites (DOPAC, HVA) in the striatum, which was accompanied by neuroinflammation and cytokine production. P2Y12R deficient mice displayed a protective effect against the neurodegenerative cell loss, the development of neuroinflammation or progression of Parkinson's disease *in vivo*. Intrathecal treatment 18 hours prior to MPTP with PSB-0739, a selective blocker of P2Y12R, which is impermeable to the blood-brain barrier, was similarly protective against the decrease in monoamine concentration, the loss of dopaminergic cells and also abolished the increase in cytokine levels in the striatum and the substantia nigra in the wild-type, but not in the P2Y12R deficient mice (Figure 5).

To further elucidate on the role of P2Y12R during PD progression, an alternative MPTP treatment model was used, which better reflect the gradual nature of neurodegeneration. Initially, MPTP was administered subchronically to promote the development of PD, and the effect of P2Y12R blockade was explored during disease progression (Tatton and Kish, 1997). Similarly to the acute administration, MPTP induced loss of dopaminergic neurons, reduction in monoamine levels and motor impairment. P2Y12R inhibition disrupted PD progression, reduced the decrease in monoamine levels, increased dopaminergic cell survival and improved motor function assessed three weeks after treatment (Figure 6).

Additionally, the intracellular mechanism underlying the protective effect of P2Y12R inhibition during PD was explored. P2Y12Rs are predominantly expressed on microglia in the central nervous system (Hodge et al., 2019). MPTP induced neuroinflammation and microglia

activation was validated by immunofluorescent staining of CD68. The increased number of CD68 positive microglia after MPTP treatment was markedly reduced, when P2Y12R were pharmacologically inhibited. No changes could be observed in P2Y12R fluorescence intensity between control, MPTP treated or MPTP combined with PSB-0739 treated groups. The activation of p38 mitogen activated protein kinase (p38 MAPK) can directly promote or can act indirectly via MK2 or MSK1/2 to stimulate cytokine production in microglia. Pharmacological inhibition of P2Y12R by PSB-0739 or the blockade of the downstream intracellular effector, ROCK by Y-27632, markedly decreased ADP induced p38 MAPK phosphorylation. These results indicate that the P2Y12R–RhoA-ROCK axis is responsible for the ADP induced activation of p38 MAPK, and imply a similar underlying intracellular mechanism during the neurodegenerative PD (Figure 7).

In conclusion we have identified the Gi coupled P2Y12 receptor to sense the increased levels of nucleotides released during cellular damage, and to initiate cytokine production via PKA RhoA ROCK pathway to regulate p38 MAPK activity. Blockade of P2Y12R prevents MPTP induced dopaminergic neuron loss and the development of Parkinson's disease; furthermore, inhibition of the receptor abrogates disease progression, reduces motor function impairment and mitigates neuronal cell death. The specific function and expression of P2Y12R proposes a promising pharmacological target to cease neuroinflammation and halt disease progression during Parkinson's disease. The manuscript detailing our results is ready for submission (**Iring A, Baranyi M, Otrokocsi L, Varga B, Gölöncsér F, Tóth A**, Bereczki D, Dénes Á, **Sperlágh B.** Inhibition of microglial P2Y12R is protective in a MPTP-induced Parkinson disease model in mice to Nature Communications).

We have also published two review articles on the topic of purinergic signaling, microglia and CNS pathology (**Calovi S, Mut-Arbona P, Sperlágh B**. Microglia and the Purinergic Signaling System. Neuroscience. 405:137-147, 2019, **Huang L, Otrokocsi L, Sperlágh B**. Role of P2 receptors in normal brain development and in neurodevelopmental psychiatric disorders. Brain Res Bull. 151:55-64, 2019) and on purinergic signalling in Parkinson's disease (**Tóth A, Antal Z,** Bereczki D, **Sperlágh B**. Purinergic Signalling in Parkinson's Disease: A Multi-target System to Combat Neurodegeneration. Neurochem Res, 44(10):2413-2422, 2019).

Figure 5.

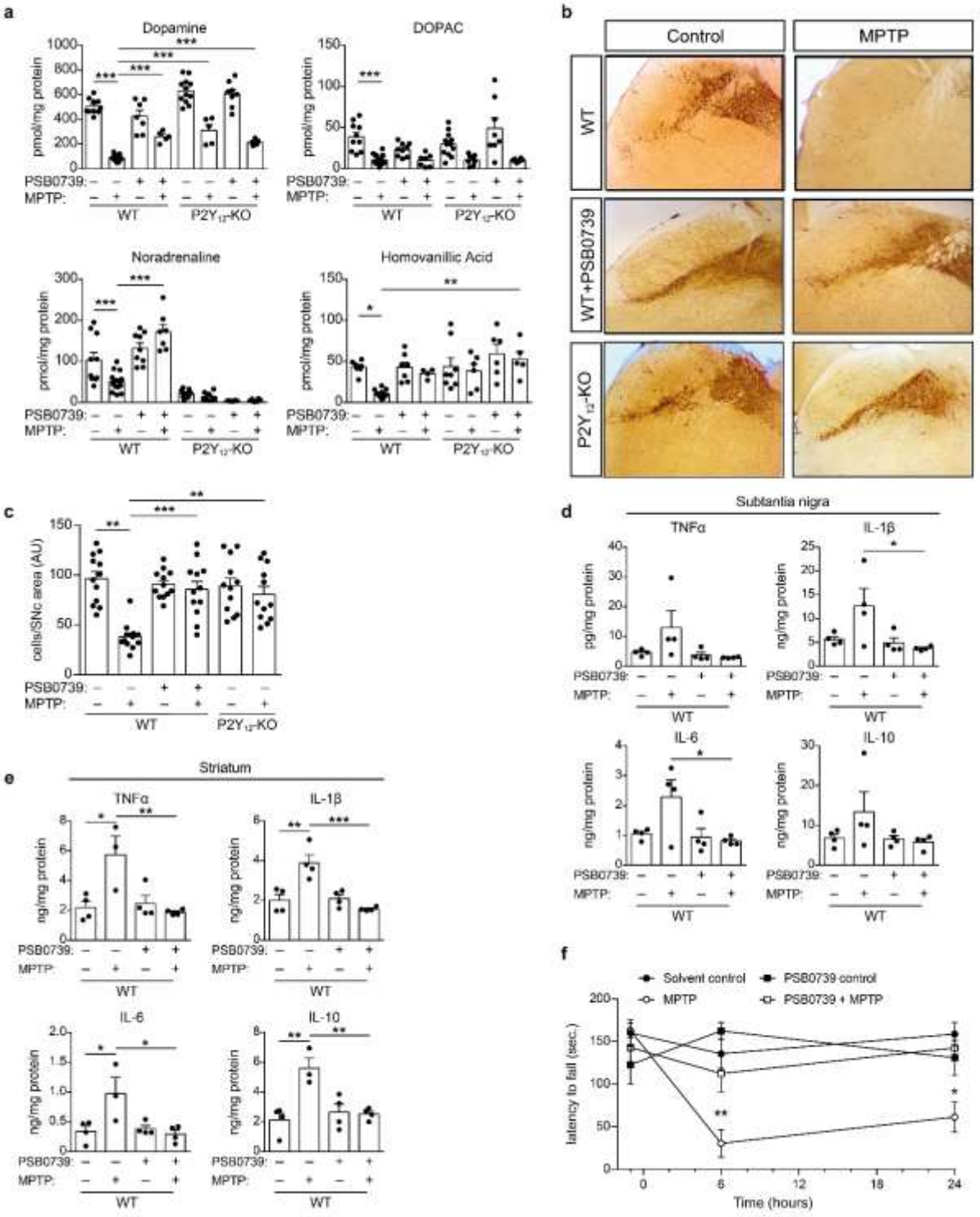

Figure 5. **P2Y12R inhibition protects against MPTP-induced PD.** (A-F) WT or P2Y12-KO mice were pretreated with 0.3 mg/kg PSB0739 or its vehicle and 4 × 20 mg/kg MPTP or its vehicle as indicated. Concentration of dopamine, DOPAC, noradrenaline and homovanillic acid were determined from striatum samples 72 hours after last MPTP administration (n=5-12) (A). Immuno-DAB staining

for TH on representative tissue sections; immunoreactivity is seen in the cell body and processes of dopaminergic and noradrenergic neurons (B); quantification of TH positive cells in the substantia nigra pars compacta (n=12) (C). TNFα, IL 1β, IL 6 and IL 10 concentration measured from substantia nigra (n=4) (D) or striatum samples (n=3-4) (E). Effect of 0.3 mg/kg PSB0739 on the motor performance during MPTP-induced PD measured on the rotarod test (n=8) (F). Data represent the mean ± SEM; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ (two-way ANOVA, with Bonferroni's post-hoc test (A,C,F), one-way ANOVA with Tukey's post-hoc test (D,E)).

Figure 6.

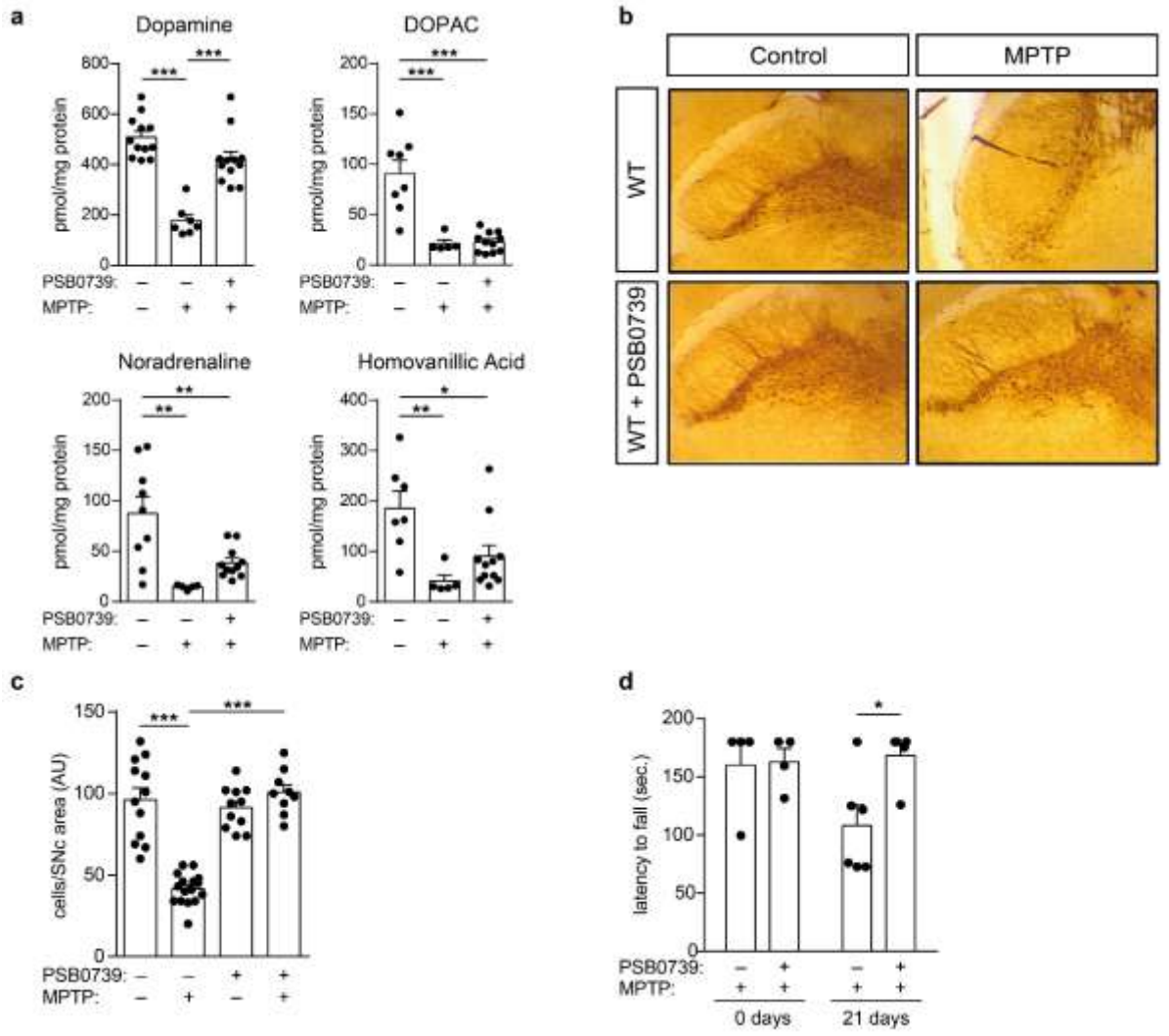

Figure 6. **P2Y12R blockade halt disease progression.** (A-D) WT mice were treated with 20 mg/kg MPTP daily for five consecutive days, followed by treatment with 0.3 mg/kg PSB0739 or its vehicle. Concentration of dopamine, DOPAC, noradrenaline and homovanillic acid were determined from striatum samples 21 days after last MPTP administration (n=5-12) (A). Immuno-DAB staining for TH on representative tissue sections; immunoreactivity is seen in the cell body and processes of dopaminergic and noradrenergic neurons (B); quantification of TH positive cells in the substantia nigra pars compacta (n=9-16) (C). Effect of PSB0739 or its vehicle on the motor performance during MPTP-

induced PD measured on the rotarod test before and 21 days after treatment (n=8) (D). Data represent the mean ± SEM; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ (one-way ANOVA, with Tukey's post-hoc test (A,C,D)).

Figure 7.

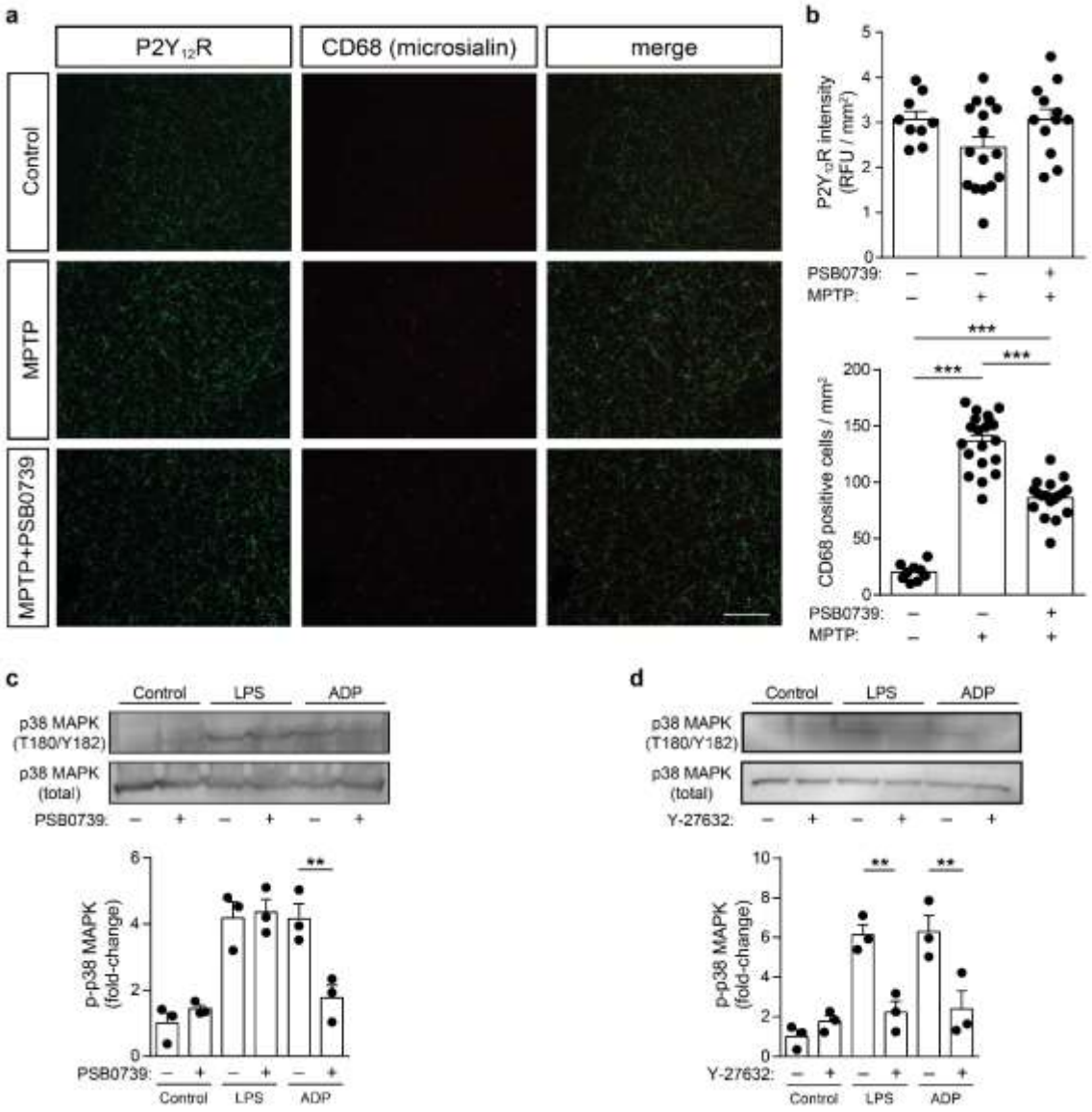

Figure 7. **P2Y12R mediates p38 MAPK phosphorylation via Rho-kinase in vitro.** (A) WT mice were treated with 20 mg/kg MPTP daily for five consecutive days, followed by treatment with 0.3 mg/kg PSB0739 or its vehicle. Shown are representative immuno-confocal microscopy images of brain slices isolated from WT mice stained with antibodies directed against P2Y12R (green), CD68 (microsialin, red) and overlay image (merge). Scale bar: 100 µm. (B) Quantification of P2Y12R fluorescence intensity (upper panel) and CD68+ cells (lower panel) (n=9-16, upper panel; n= 9-20, lower panel). (C-D) Murine BV2 microglia cells were pretreated with PSB0739 (500 nM, 30 min) (C) or with Y-27632 (10 µM, 30 min) (D) and were incubated with LPS (100 ng/mL, 60 min), ADP (10

μM, 5 min) or solvent (control) and phosphorylation of p38 MAPK at threonine 180 / tyrosine 182 were determined by immunoblotting. Graphs show the densitometric evaluation (n=3). Data represent the mean ± SEM; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ (one-way ANOVA, with Tukey's post-hoc test (B), two-way ANOVA with Bonferroni's post-hoc test (C,D)).

5. Regulation of depressive–like behaviour by P2Y12 receptors

In this study, we investigated the potential role of P2Y12R in a mouse model of depressive-like behaviors according to our previous study (Csölle et al., 2013). Wild-type (WT) C57BL/6J and P2Y12R receptor gene deficient mice (P2Y12R-/-) of C57BL/6J background were used. The effect of P2Y12R gene deficiency was examined in naïve animals subjected to automated tail suspension test (TST) (Fig. 8/A) and forced swim test (FST) (Fig. 8/B). The total immobility time in the P2Y12R-/- animals was significantly decreased compared to the WT mice. The same behavioral difference was observed in the FST, the total immobility time was markedly reduced in the P2Y12R-/- mice in comparison to the WT animals. Afterward, the animals were subjected to LPS-induced sucrose preference test. After determining the baseline sucrose consumption mice were injected with 0.2 mg/kg LPS i.p. or equal volume of saline. In the P2Y12R-/- mice the LPS injection induced anhedonia compared to the saline injected knock-out animals up to 36 hours and compared to the WT animals injected with LPS up to 60 hours. No difference was observed between the LPS and the saline injected wild type animals (Fig. 98).

Our preliminary results suggests that the P2Y12R is potentially involved in depressive-like behaviors, although its role seems to be dependent on the test used. Ongoing studies are performed to fully elucidate the role of P2Y12R in the pathogenesis of depression-like behaviour, and we plan to publish these results after completing these steps.

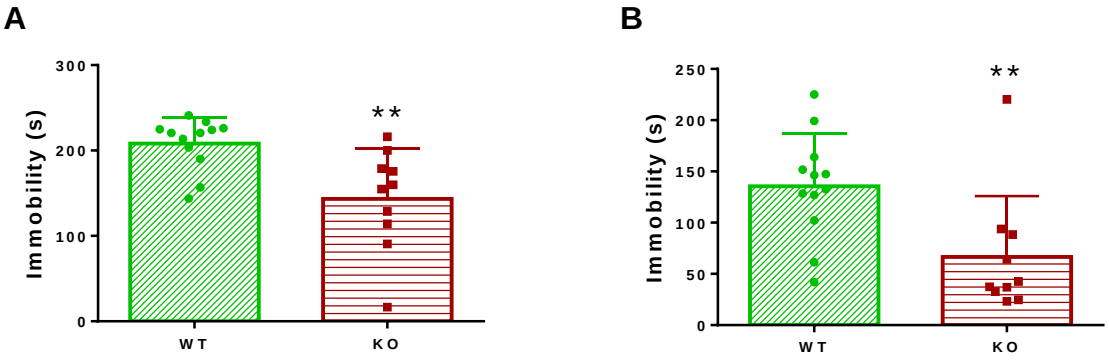

Figure 8. A and B: **Automated tail suspension test (TST) and forced swim test (FST).** The genetic deletion of P2Y12R decreases the basal immobility of the animals in the TST and in the FST. Immobility time is expressed in seconds. The total duration of each test was 360 seconds. WT: wild type mice (n=12), KO: P2Y12R knock-out mice (n=10); Data expressed as mean±SEM; Mann-Whitney t-test, **: $p<0.01$

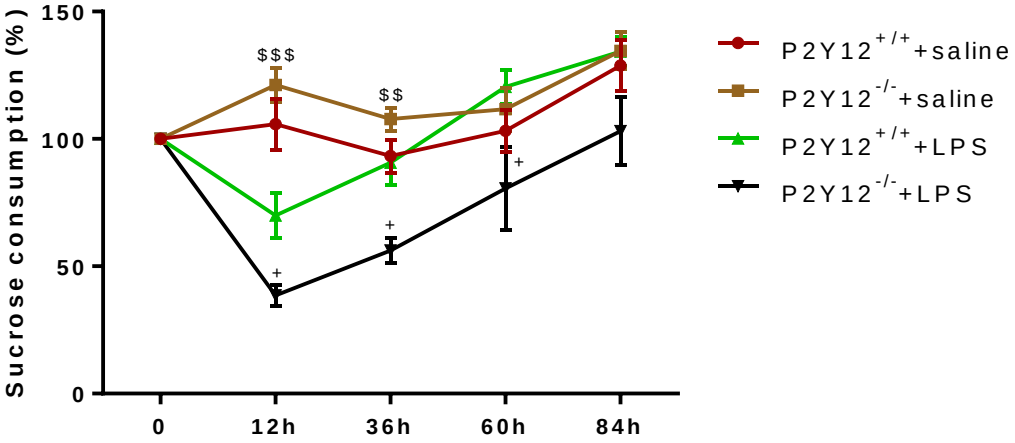

Figure 9. **Sucrose preference test.** LPS-administration induced decrease in sucrose preference was augmented in the P2Y12 knock-out mice as a sign of anhedonia. Baseline sucrose intake was determined in a 3-days habituation period before LPS injection given as 100%. Mice were administered with 0.2 mg/kg LPS or equal volume of saline. The sucrose consumption was expressed as a percentage of the baseline (%). $P2Y12^{+/+}$+saline: n=6, $P2Y12^{-/-}$+saline: n=5, $P2Y12^{+/+}$+LPS: n=6, $P2Y12^{-/-}$+LPS: n=5; data expressed as mean±SEM; 3-way ANOVA with Newman-Keuls post-hoc test; +: $p<0.05$ $P2Y12^{+/+}$+LPS vs. $P2Y12^{-/-}$+LPS; $$$: $p<0.001$, $$: $p<0.01$ $P2Y12^{-/-}$+saline vs. $P2Y12^{-/-}$+LPS

6. The involvement of P2Y12R in microglia phagocytosis and in the regulation of adult hippocampal neurogenesis

During adult hippocampal neurogenesis, the majority of newborn cells undergo apoptosis and are rapidly phagocytosed by resident microglia to prevent the spillover of intracellular contents. In the CNS, P2Y12R is expressed predominantly by the microglia and is the part of the unique human microglia signature. In this study, we examined the role of P2Y12R in microglia phagocytosis and the participation of microglia in the regulation of adult hippocampal neurogenesis, in collaboration with the group of Amanda Sierra, Achucarro Basque Center for Neuroscience, Leioa, Bizkaia, Spain. To this end, we have explored microglia phagocytosis and adult neurogenesis in the subgranular zone of the hippocampus in mice chronically deficient for three distinct microglial phagocytosis pathways (P2Y12R, MerTK/Axl (tyrosine kinases of the TAM family, which bind to phosphatidylserine adapter/bridging molecules Growth arrest specific factor 6 (Gas6) and Protein S (Pros1)and GPR34. All these receptors have been described to actively participate in phagocytosis (Elliott et al., 2009; Fourgeaud et al., 2016; Preissler et al., 2015). First, we assessed phagocytosis in the hippocampus by quantifying the Ph index (the percentage of apoptotic cells engulfed by microglia), which is around 90% in physiological conditions, and found significantly lower Ph index in the three KO models (74.8 ± 0.9% for P2Y12, 61.5 ± 1.6% for MerTK/Axl, 67.7 ± 3.4% for GPR34 KO mice). In addition, the microglial Ph capacity (weighted average of the number of pouches containing apoptotic cells per microglia, i.e., the average number of phagocytic pouches per microglia) was significantly reduced in the three KO models.

Next, we studied hippocampal neurogenesis in these phagocytosis impaired KO models and observed that all three showed a significant decrease in the population of neuroblasts, labeled with doublecortin (DCX). In addition, we assessed proliferation by injecting mice with bromodeoxyuridine (BrdU, 150mg/kg, 24h), an analogue of thymidine incorporated during S phase in dividing cells); or with the proliferation marker Ki67+. P2Y12 KO mice presented the

most profound decrease in both neuroblasts (reduction of 31.7 ± 2.7%) and neuroblast proliferation (reduction of 39.3 ± 9.8%). We further studied the formation of newborn neurons using BrdU in this model. Four weeks after the BrdU injection, when the mice were 2mo, both total BrdU+ cells and newborn neurons (NeuN+/BrdU+) were reduced in P2Y12 KO mice compared to WT mice (reduction of 24.6 ± 9.7%), in parallel to a decrease in phagocytosis (Ph index and Ph capacity).

To confirm the specificity of these results, we analyzed the expression of P2Y12, GPR34, MerTK and Axl in FACS sorted cells from 1m mice. We found that, with the exception of Axl, all these receptors were selectively expressed in microglia, suggesting that the disruption of neurogenesis in the KO models might be attributable to the lack of these receptors in microglia.

We then followed an in vitro approach to perform a transcriptomic analysis of microglial phagocytosis and identified genes involved in metabolism, chromatin remodeling, and neurogenesis-related functions. Finally, we determined that the secretome of phagocytic microglia limits the production of new neurons both in vivo and in vitro. Our data suggest that reprogrammed phagocytic microglia acts as a sensor of local cell death, modulating the balance between cell proliferation and cell survival in the neurogenic niche, supporting the long-term maintenance of adult hippocampal neurogenesis. Our group has contributed to this study with BrdU+ studies in P2Y12 KO mice. These data are published (Diaz-Aparicio I, Paris I, Sierra-Torre V, Plaza-Zabala A, Rodríguez-Iglesias N, Márquez-Ropero M, Beccari S, Huguet P, Abiega O, Alberdi E, Matute C, Bernales I, Schulz A, **Otrokocsi L**, **Sperlagh B**, Happonen KE, Lemke G, Maletic-Savatic M, Valero J, Sierra A. Microglia Actively Remodel Adult Hippocampal Neurogenesis through the Phagocytosis Secretome. J Neurosci. 2020 Feb 12;40(7):1453-1482.).

7. The key role of microglia P2Y12 receptor in the spread of the neurotropic virus infection

In the framework of another, in-house collaboration with the Laboratory of Neuroimmunology, IEM (Ádám Dénes and his group) we have studied the contribution of microglia P2Y12 receptors to the microglia recruitment following virus infection. Using a well-established model of alpha herpesvirus infection that reaches the brain exclusively via retrograde trans synaptic spread from the periphery, this study showed that microglia are recruited to and isolate infected neurons within hours. Selective elimination of microglia resulted in a marked increase in the spread of infection and egress of viral particles into the brain parenchyma, which are associated with diverse neurological symptoms. Microglia recruitment and clearance of infected cells required cell-autonomous P2Y12 signalling in microglia, triggered by nucleotides released from affected neurons. In contrast, we also identified microglia as key contributors to monocyte recruitment into the inflamed brain, but this process was largely independent of P2Y12. P2Y12-positive microglia are also recruited to infected neurons in the human brain during viral encephalitis and both microglial responses and leukocyte numbers correlate with the severity of infection. The specific contribution of my group to this study was to provide the P2ry12-/- mouse colony for the experiments, to perform the nucleotide analyses by HPLC analysis in the supernatant and in the cellular fraction of control and virus-infected neuronal cultures, and to perform ecto-ATPase enzyme histochemistry. ATP, but not ADP, AMP and adenosine levels were significantly higher in conditioned media collected from virus-infected cultures, indicating an ATP-rich extracellular milieu under these conditions. Densitometric analysis revealed that the ecto-ATPase activity is also significantly enhanced upon infection. (Figure 4 a,b in the published article).

In conclusion, these data identified a key role for microglial P2Y12 in defence against neurotropic viruses. The results are published. (Fekete R, Cserép C, Lénárt N, Tóth K, Orsolits

B, Martinecz B, Méhes E, Szabó B, Németh V, Gönci B, **Sperlágh B**, Boldogkői Z, **Kittel Á, Baranyi M**, Ferenczi S, Kovács K, Szalay G, Rózsa B, Webb C, Kovacs GG, Hortobágyi T, West BL, Környei Z, Dénes Á. Microglia control the spread of neurotropic virus infection via P2Y12 signalling and recruit monocytes through P2Y12-independent mechanisms. Acta Neuropathol. 2018 Sep;136(3):461-482)

8. Microglia monitor and protect neuronal function through specialized somatic purinergic junctions via P2Y12 receptors

As a part of the aforementioned collaboration, we have identified an interaction site between neuronal cell bodies and microglial processes in mouse and human brain. Somatic microglia-neuron junctions have a specialized nanoarchitecture optimized for purinergic signaling. Activity of neuronal mitochondria was linked with microglial junction formation, which was induced rapidly in response to neuronal activation and blocked by inhibition of P2Y12 receptors. Brain injury-induced changes at somatic junctions triggered P2Y12 receptor-dependent microglial neuroprotection, regulating neuronal calcium load and functional connectivity. Thus, microglial processes at these junctions could potentially monitor and protect neuronal functions. The specific contribution of our group to this study was to provide the p2ry12-/- mouse colony for the experiments and to measure extracellular ATP levels in response to KCl depolarization in a primary microglia-neuron culture. K^+ depolarization induced a robust release of ATP which was not sensitive to the inhibitors of N and L-type voltage sensitive Ca^{2+} channels, but was inhibited by nimodipine, the L-Type Ca^{2+} channel blocker or the vesicular nucleotide transporter inhibitor clodronate (Figure 3J in the published article). These results are published in Science (Cserép C, Pósfai B, Lénárt N, Fekete R, László ZI, Lele Z, Orsolits B, Molnár G, Heindl S, Schwarcz AD, Ujvári K, Környei Z, Tóth K, Szabadits E, **Sperlágh B, Baranyi M,** Csiba L, Hortobágyi T, Maglóczky Z, Martinecz B, Szabó G, Erdélyi F, Szipőcs R, Tamkun MM, Gesierich B, Duering M, Katona I, Liesz A, Tamás G, Dénes Á. Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. Science. 367(6477):528-537, 2020).

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