UCSF
UC San Francisco Electronic Theses and Dissertations

Title
The ADP receptor P2Y12 senses tissue injury

Permalink
https://escholarship.org/uc/item/2ns8514b

Author
Hollopeter, Gunther

Publication Date
2005

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

The ADP Receptor $P2Y_{12}$ Senses Tissue Injury

by

Gunther Hollopeter

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

UCSF LIBRARY

Copyright 2005

by

Gunther Hollopeter

Acknowledgments

This work captures the cooperative efforts of many talented scientists. I would first like to thank David Julius for providing a fruitful research environment. He has a keen sense of what is interesting, important, and creative. I have learned more in graduate school under his tutelage than I ever imagined possible. Next I would like to thank Pam Conley for bringing this project into the lab and being such a wonderful collaborator. Huai-Hu Chuang helped me to characterize the positive clone and I often wonder if he is the brightest person I know. Sharon Haynes carried out many of the experiments in chapter 3 and I truly appreciate her helping me to investigate the role of $P2Y_{12}$ in microglia. This has enabled me to (unwisely?) spend time pursuing another (crazy?) project in the lab. I will always remember her as the embodiment of innate kindness and generosity. Additionally I would like to thank Jeannie Poblete for perpetually dropping whatever she was doing to help me (talk about innate kindness and generosity!) Pam Tsuruda is also one of those people that only know how to give. I have found her a constant source of advice, brainstorming, assistance, conversation, and fun. I thank my parents, Alexia and Wayne, for always expecting the best of me and valuing education. They are consistently encouraging, supportive and loving. I am amazed at my parent's total and complete dedication to their offspring. Carrie Adler has also shown a level of trust, dedication and love that is inspiring. I am honored that she would chose to share her unique perspective on the world with me. My life is enriched many fold because of it.

UCSF LIBRARY

Permission

From: J.Rutt@nature.com

Subject: RE: doi: 10.1038/35051599

Date: September 7, 2005 3:02:00 AM PDT

To: ghol1070@itsa.ucsf.edu

Dear Gunter,

Thank you for your request to reproduce the following letter, published in Nature (referenced below), for your thesis.

Nature 409, 202-207 (11 January 2001) | Identification of the platelet ADP receptor targeted by antithrombotic drugs

I am pleased to grant your permission for one-time use, in print, microfilm and on the world wide web, with the following specifications:

(a) The paper is to be used for display/educational purposes only and will not be used to generate profit.

(b) The journal's name and the authors will be properly cited.

(c) In cases of internet usage, a link will be provided from the thesis homepage (please email the URL for your organization's website) to www.nature.com/nature

The permission statement must appear in the same field of vision as the material used. Any further usage will be requested in writing.

Please do not hesitate to contact me with any further questions.

Best wishes,

Jessica Rutt

UCSF LIBRARY

Nature Permissions Officer

The Macmillan building

4-6 Crinan Street

London

N1 9XW

Tel: 44 (0) 207 833 4000

Fax: 44 (0) 207 843 4596

permissions@nature.com

http://www.nature.com

UCSF LIBRARY

Advisor Statement

Gunther Hollopeter completed the following:

Chapter 2

Figure 1

Figure 2

Figure 4 C

Figure 5 C

Chapter 3

Figure 2 B and C

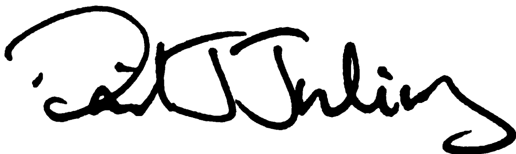
Gunther Hollopeter assisted Sharon Haynes in completing the following:

Chapter 3

Figure 2 A and D

Figure 4

This work is constitutes a body of work sufficient to satisfy the requirements for a doctoral degree awarded from the University of California, San Francisco.



David Julius

Professor of Cellular and Molecular Pharmacology

UCSF LIBRARY

Abstract

The ADP Receptor $P2Y_{12}$ Senses Tissue Injury

Gunther Hollopeter

One characteristic of self-sustaining life is the ability to detect and respond to injury. Following cellular damage, local release of nucleotides may serve as an important signal to inform nearby cells and initiate corrective responses. Several receptor sites for purine nucleotides have been identified, including a G_i coupled receptor for ADP hypothesized to promote platelet aggregation and microglia chemotaxis. We purified a cDNA encoding this receptor, termed $P2Y_{12}$, from rat platelet mRNA using a functional expression assay in frog oocytes and observe receptor transcripts in platelets and microglia. We show that $P2Y_{12}$ is inactivated by cysteine modification and genetic loss of $P2Y_{12}$ is associated with a human bleeding disorder. We also note that the robust expression in microglia is reduced following activation with bacterial endotoxin. Microglia of $P2Y_{12}$ deficient mice lack the normal chemotropic response to ADP *in vitro* and *in vivo*. Compared to microglia of wild type mice, those of $P2Y_{12}$ knockout mice are slow to extend processes towards the site of cortical damage induced with a laser. These data suggest that $P2Y_{12}$ is required in order for both platelets and microglia to properly respond to tissue injury.

UCSF LIBRARY

Table of Contents

CHAPTER 1 1

Introduction

CHAPTER 2 10

Molecular Identification of the Platelet ADP Receptor Targeted by Antithrombotic Drugs

CHAPTER 3 37

The $P2Y_{12}$ Receptor Regulates Microglial Activation by Extracellular Nucleotides

CHAPTER 4 66

Future Directions

UCSF LIBRARY

List of Figures

Chapter 2

Figure 1 $P2Y_{12}$ is a G protein–coupled receptor that responds to ADP......32

Figure 2 Currents stimulated by ADP in oocytes expressing $hP2Y_{12}$ with Kir3.1 and 3.4 are inhibited by 2MeSAMP, C1330-7 and a thiol reagent.......33

Figure 3 Activation of $hP2Y_{12}$ in CHO cells inhibits adenylyl cyclase........34

Figure 4 $P2Y_{12}$ receptor is selectively expressed in platelets and brain.....35

Figure 5 A frame-shift mutation within the $hP2Y_{12}$ gene is associated with a bleeding disorder.......36

Chapter 3

Figure 1 $P2Y_{12}$ immunoreactivity is localized to the cell membrane of microglia in the brain.......60

Figure 2 $P2Y_{12}$ expression is diminished in activated microglia..............61

Figure 3 Nucleotide-evoked membrane ruffling and chemotaxis are abolished in microglia lacking the $P2Y_{12}$ receptor.....................62

Figure 4 Loss of $P2Y_{12}$ receptors abrogates response of microglia to ATP injection or focal laser ablation in the living brain..............63

Figure S1 Microglia from $P2Y_{12}$-deficient mice have normal morphology and prevalence within the CNS.......64

Figure S2 Microglia express $P2Y_{13}$ transcripts, but not detectable receptor protein.......65

UCSF LIBRARY

CHAPTER 1

Introduction

UCSF LIBRARY

INTRODUCTION

One of the key facets of self-sustaining life is the ability to recognize and repair injury. Cellular damage is an inevitable consequence of organisms interacting with their environment and mechanisms have arisen to detect and respond to injury. In complex multicellular organisms, specialized cells of the nervous system are designed to sense tissue damage and promote withdrawal and/or avoidance of the injurious situation. In addition, there are specialized cells of the cardiovascular and immune systems that are geared towards detection of local tissue injury and subsequent repair. In general, their job descriptions include locating the site of damage, prevention of further injury, clearing cellular debris, and promoting tissue repair. Most of the time, these sentinels of the body perform their tasks efficiently, however, inappropriate activation of these cells can lead to a multitude of pathological conditions. Thus, understanding the steps regulating their activation is of great benefit for controlling human disease states.

Nucleotides such as ATP and ADP may represent key elements utilized by cells to detect injury. All cells use nucleotides as building blocks for macromolecules (namely genetic material), second messenger systems and as the major energy currency. Thus, all cells contain millimolar concentrations of nucleotides. Cellular trauma that results in loss of membrane integrity will liberate a substantial quantity of these ubiquitous cytoplasmic components and serve to inform local cells of the damage[1]. In addition, nucleotides are released in a

regulated fashion from several cell types including platelets, neurons and astrocytes[2]. Release of nucleotides by these cells, especially platelets, is induced by injury such as vascular damage[3]. Therefore, release of nucleotides into the extracellular space is a signal that could enable specialized injury detector cells to localize the damage site.

It has been noted for over half a century that nucleotides exert diverse actions on a variety of cell types[4]. For example, application of nucleotides regulates physiological responses of the cardiovascular, immune, and nervous systems[5]. Using a number of structurally distinct pharmacological substances in combination with physiology assays, ATP receptor sites (P2 purinergic) have been classified into two main categories. The P2Y receptors mediate their effects via the activation of G proteins and are referred to as metabotropic receptors[6]. Ion channels that are opened by extracellular nucleotides are classified as P2X ionotropic receptors[7]. The molecular identification of a P2Y receptor from a neuroblastoma-glioma cDNA library using an expression cloning strategy in *Xenopus* oocytes validated the theory that nucleotides are indeed used as extracellular signaling molecules and provided a genetic handle on the receptor site predicted by pharmacology[8]. Using sequence homology, a number of related P2Y receptors have been cloned from many different vertebrates[9]. The majority of these P2Y receptors are widely expressed and couple to Gq type G proteins leading to subsequent release of calcium from intracellular stores[10]. The first P2X receptor was also cloned using a functional expression screen in

Xenopus oocytes[11]. This ATP receptor, along with additional P2X receptors defines a novel class of ligand-gated ion channels with two transmembrane segments. The P2X class of receptors is now a large and diverse family whose members are proposed to mediate a variety of physiological processes[12]. The cloning and characterization of purinergic receptors has solidified the idea that extracellular nucleotides can, indeed, serve as autocrine and paracrine signals.

Within the cardiovascular system one of the major sites of nucleotides action is the platelets. These thrombocytes are small, enucleated cells produced in the bone marrow that circulate through the bloodstream. They are important for the blood clotting process following injury. At sites where bleeding occurs, they swell to plug the damaged vessel by adhering to one another and the vascular wall. It has been appreciated for years that dispersed platelets found in plasma collected from blood will exhibit clotting characteristics *in vitro* upon application of ADP. Additionally, platelets themselves release ADP from their dense granule type vesicles in response to ADP and other platelets activators. Thus ADP exerts a powerful positive feedback effect on platelets[13]. The pharmacology and signaling mechanism of this ADP response has been well characterized. It is now recognized that a large component ADP's action on platelets is mediated by a P2Y receptor that couples exclusively to the inhibition of adenyl cyclase through activation of pertussis toxin-sensitive Gi proteins[14]. This receptor is quite medically relevant because some of the best antithrombotic drugs currently available, primarily clopidogrel and ticlopidine, are thought to act by covalently

inactivating this site on platelets[15]. Clopidogrel (trade name, Plavix) is prescribed for patients with an established propensity to form clots such as a recent history of myocardial infarction or stroke. For such patients, this drug will reduce the chances of future thrombotic events. However, it has been associated with an increased tendency to bleed, and rarely, with a serious medical condition known as thrombotic thrombocytopenic purpura[16]. Thus, it is important to better understand how this ADP receptor regulates platelet function and to develop reversible inhibitors of its actions. The molecular identification of this receptor will serve as an important steppingstone toward accomplishing these goals.

Within the central nervous system (CNS), nucleotides exert effects on microglia. These immune cells of the brain, like platelets, are derived from the bone marrow and respond to tissue injury[17]. However, they are more poorly understood. Microglia are believed to sit quietly in an inactive state until the CNS becomes damaged due to injury or disease. At that time, their number increases in the vicinity of CNS damage where they exhibit a macrophage-like phenotype[18]. It remains controversial as to whether they are helpful or harmful to the survival of neurons and supporting cells at sites of microglial activation[19]. It is known however, that microglia express a variety of nucleotide receptors including members of both the P2Y and P2X class[20]. Additionally, a multitude of effects have been noted following application of nucleotides to microglia in culture and *in vivo*. As such, it is well accepted that microglia are uniquely tuned to detect extracellular nucleotides. Interestingly, microglia in culture are reported to

UCSF LIBRARY

change their morphology and move towards ATP and ADP[21]. This is hypothesized to be the mode by which microglia locate sites of nucleotide release *in vivo*, such as may occur at injury sites. It is worth noting that the pharmacological profile of this microglial response shares striking similarity with that noted for the ADP receptor site on platelets. However, the paucity of selective purinergic receptor antagonists for *in vivo* studies makes it difficult to establish a role for nucleotides in regulating microglia physiology within the CNS. Nor is it possible to determine if the ADP receptor expressed by platelets is similar not only functionally, but also molecularly, without a genetic handle on the receptor site. The experiments described herein are the results of a project initiated by COR Therapeutics to identify a cDNA encoding the Gi-coupled receptor for ADP on platelets.

UCSF LIBRARY

References

1. Franke, H. & Illes, P. Involvement of P2 receptors in the growth and survival of neurons in the CNS. *Pharmacol Ther* (2005).
2. White, T. D. Role of adenine compounds in autonomic neurotransmission. *Pharmacol Ther* **38**, 129-68 (1988).
3. Hechler, B., Cattaneo, M. & Gachet, C. The P2 receptors in platelet function. *Semin Thromb Hemost* **31**, 150-61 (2005).
4. Drury, A. N. & Szent-Gyorgi, A. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J Physiol* **68**, 213-37 (1929).
5. Burnstock, G. Overview. Purinergic mechanisms. *Ann N Y Acad Sci* **603**, 1-17; discussion 18 (1990).
6. O'Connor, S. E., Dainty, I. A. & Leff, P. Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol Sci* **12**, 137-41 (1991).
7. Bean, B. P. Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol Sci* **13**, 87-90 (1992).
8. Lustig, K. D., Shiau, A. K., Brake, A. J. & Julius, D. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci U S A* **90**, 5113-7 (1993).
9. Burnstock, G. Introduction: P2 receptors. *Curr Top Med Chem* **4**, 793-803 (2004).

10. Harden, T. K., Boyer, J. L. & Nicholas, R. A. P2-purinergic receptors: subtype-associated signaling responses and structure. *Annu Rev Pharmacol Toxicol* **35**, 541-79 (1995).

11. Brake, A. J., Wagenbach, M. J. & Julius, D. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371**, 519-23 (1994).

12. North, R. A. Molecular physiology of P2X receptors. *Physiol Rev* **82**, 1013-67 (2002).

13. Mills, D. C. ADP receptors on platelets. *Thromb Haemost* **76**, 835-56 (1996).

14. Daniel, J. L. et al. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* **273**, 2024-9 (1998).

15. Gachet, C. et al. ADP receptor induced activation of guanine nucleotide binding proteins in rat platelet membranes--an effect selectively blocked by the thienopyridine clopidogrel. *Thromb Haemost* **68**, 79-83 (1992).

16. Bennett, C. L. et al. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N Engl J Med* **342**, 1773-7 (2000).

17. Kreutzberg, G. W. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* **19**, 312-8 (1996).

18. Stence, N., Waite, M. & Dailey, M. E. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* **33**, 256-66 (2001).

UCSF LIBRARY

19. Chao, C. C., Hu, S., Molitor, T. W., Shaskan, E. G. & Peterson, P. K. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol* **149**, 2736-41 (1992).

20. Inoue, K. Microglial activation by purines and pyrimidines. *Glia* **40**, 156-63 (2002).

21. Honda, S. et al. Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. *J Neurosci* **21**, 1975-82 (2001).

UCSF LIBRARY

CHAPTER 2

Molecular Identification of the Platelet ADP Receptor Targeted by Antithrombotic Drugs

reprinted with permission from Nature 409, 11 January 2001

UCSF LIBRARY

Molecular Identification of the Platelet ADP Receptor Targeted by Antithrombotic Drugs

Gunther Hollopeter[1], Hans-Michael Jantzen[2], Diana Vincent[2], Georgia Li[2,3], Laura England[1,4], Vanitha Ramakrishnan[2], Ruey-Bing Yang[2], Paquita Nurden[5], Alan Nurden[5], David Julius[1] and Pamela B. Conley[2]*

[1]Department of Cellular & Molecular Pharmacology and Program in Neuroscience, University of California, San Francisco, San Francisco, CA 94143

[2]COR Therapeutics, Inc., South San Francisco, CA, 94080

[3] present address, Advanced Medicine, South San Francisco, CA 94080

[4] present address, FibroGen, South San Francisco, CA 94080

[5]UMR 5533 CNRS, Hôpital Cardiologique, 33605 Pessac, France

*to whom correspondence should be addressed. E-mail:pconley@CORR.com

UCSF LIBRARY

Platelets play a critical role in the maintenance of normal hemostasis, and perturbations of this system can lead to pathological thrombus formation and vascular occlusion, resulting in stroke, myocardial infarction and unstable angina. ADP released from damaged vessels and red blood cells induces platelet aggregation through activation of the integrin GPIIb-IIIa and subsequent fibrinogen binding. ADP is also secreted from platelets upon activation, providing a positive feedback loop that potentiates the actions of many platelet activators [1]. ADP mediates platelet aggregation through its action on two G protein coupled receptor (GPCR) subtypes [2,3]. The P2Y$_1$ receptor couples to G$_q$ and mobilizes intracellular calcium to mediate platelet shape change and aggregation [4, 5]. The second ADP receptor required for aggregation (variously called P2Y$_{ADP}$, P2Y$_{AC}$, P2Y$_{cyc}$ or P2T$_{AC}$) is coupled to the inhibition of adenylyl cyclase through G$_i$. The molecular identity of the G$_i$-linked receptor has remained elusive, even though it is the target of efficacious antithrombotic agents such as ticlopidine and clopidogrel [6-8], and AR-C66096 [9]. Here we describe the cloning of this receptor, designated P2Y$_{12}$, and show that a patient with a bleeding disorder [10] is defective in this gene. Cloning of the P2Y$_{12}$ receptor should facilitate the development of better antiplatelet agents to treat a variety of cardiovascular diseases.

UCSF LIBRARY

To identify the G_i-linked platelet ADP receptor, we engineered *Xenopus* oocytes to allow detection of G_i-linked responses through a sensitive electrophysiological assay. This strategy is based on the fact that several G_i-coupled receptors, such as the m2 muscarinic receptor, release $G\beta\gamma$ subunits from heterotrimeric G proteins, thereby activating inwardly rectifying potassium (K^+) channels (Kir3.1-4)[11]. A cDNA library from rat platelets was screened in oocytes expressing Kir3.1 and 3.4 and three positive pools that responded to 10 μM ADP (as determined by an increase in K^+ current) were identified. Subfractionation of one of these pools led to the identification of a single clone tentatively designated as $P2Y_{12}$. The current induced by ADP was K^+-dependent since replacement of K^+ in the bath solution resulted in a complete loss of current (Fig 1A). Additionally, injection of Kir or $P2Y_{12}$ cRNAs alone gave no ADP-dependent currents, indicating that the observed signal was not due to activation of an endogenous purinergic receptor and was Kir-dependent (Fig 1B). Moreover, when cRNA encoding pertussin toxin was injected together with the rat $P2Y_{12}$ clone, the response to ADP was abolished (Fig 1B), as predicted for the G_i-linked platelet ADP receptor [12]. The human $P2Y_{12}$ homolog was isolated from a human platelet library and similar results were obtained when this cRNA was expressed in *Xenopus* oocytes (Fig 1A,B).

One hallmark of the G_i-linked platelet ADP receptor is that substitution of alkylthio groups at the 2-position of the adenine ring increases potency at the receptor [1,13,14]. Consistent with this, 2MeSADP displayed 2 orders of magnitude greater potency compared to ADP (with an EC50 of 0.9 nM and 300 nM,

respectively) (Fig. 1C). In contrast, other nucleoside or nucleotide derivatives were without effect (Fig 1B). We also examined the actions of several antagonists specific for the platelet G_i-linked ADP receptor. Treatment of *Xenopus* oocytes expressing the rat or human $P2Y_{12}$ receptor with the nucleotide derivative 2MeSAMP [2] or a non-nucleotide inhibitor C1330-7[15], blocked ADP-induced K^+ currents with IC50's of 1.4 μM and 40 nM, respectively (Fig 2B). In contrast, the $P2Y_1$-selective antagonist A3P5P [16] had no inhibitory effect on the signal evoked by ADP at the rat or human $P2Y_{12}$ (Fig 2A). Thus, when expressed in *Xenopus* oocytes, the $P2Y_{12}$ receptor recapitulates the pharmacological profile previously described for the platelet G_i-linked ADP receptor. The only anomaly that we observed relates to the action of ATP-αS, which behaved as a weak agonist, rather than an antagonist at the cloned receptor. This finding is somewhat unexpected since ATP derivatives reportedly antagonize the platelet G_i-linked receptor. However, this discrepancy may reflect partial degradation or impurities in commercially available preparations of ATP-αS, or differences between the platelet and oocyte environments, such as the degree of ectonucleotidase activity. Indeed, recombinant $P2Y_1$ receptors respond differentially to ATP, depending on the expression system utilized [17, 18].

Chinese Hamster Ovary (CHO) cells expressing the $hP2Y_{12}$ receptor displayed ADP-mediated repression of forskolin-stimulated cAMP levels in a dose-dependent manner, reaching a maximum of 47% reduction at 10 μM ADP (Fig 3A). The repression of cAMP levels by 1 μM ADP was reversed by the selective antagonists 2MeSAMP and C1330-7 (Fig 3B), in agreement with the

UCSF LIBRARY

pharmacological profile observed in *Xenopus* oocytes, and as described for the G_i-coupled receptor on platelets. Neither of these antagonists had effects on forskolin-stimulated cAMP levels in the absence of agonist. Similar responses to ADP were observed in rat 2-9 fibroblasts stably expressing $rP2Y_{12}$ (data not shown). Pretreatment of transfected cells with pertussis toxin abolished ADP effects on forskolin-stimulated cAMP (Fig 3C), suggesting that the response is G_i-mediated.

Northern blot analysis demonstrated that $P2Y_{12}$ is abundantly expressed in human platelets, and to a lesser extent in brain (Fig. 4A, B). The predominant transcript of 2.4 kb was absent from all other tissues examined, including peripheral blood leukocytes. A fainter species of ~4.5 kb was also detected in platelet and brain, while a prominent band of ~1.0 kb (Fig 4B) was observed only in platelet RNA. Among rat tissues, selective expression in platelets and brain was also seen (data not shown). Thus, the mRNA for this novel GPCR has a restricted expression pattern and is abundantly present in platelets, consistent with this cDNA encoding the platelet G_i-linked receptor. Within the brain, the 2.4 kb species was observed in numerous subregions, including the amygdala, caudate nucleus, corpus collosum, hippocampus, substantia nigra, and thalamus (data not shown). Cellular resolution of $rP2Y_{12}$ expression was obtained by *in situ* hybridization histochemistry of brain sections where punctate staining was noted throughout white and gray matter (Fig. 4C). Principal cells of the hippocampus did not stain, nor was a laminar pattern of expression observed in the neocortex. These observations are consistent with a glial expression pattern. Interestingly,

the only cell line previously described to express a P2Y purinergic receptor that is negatively coupled to adenylyl cyclase is the rat C6 glioma cell line [19]. Indeed, a 2.4 kb mRNA species was detected in these cells by Northern analysis with a $rP2Y_{12}$ probe (data not shown).

Using a rabbit polyclonal antisera directed to the predicted amino-terminus of $rP2Y_{12}$, we assessed surface expression of receptor protein on stably-transfected rat 2-9 fibroblasts or rat platelets using flow cytometry. At an antibody concentration of 25 μg/ml, a 9-fold (Fig 4E) and 4-fold (Fig 4D) increase in mean fluorescence intensity (compared to a control antibody) was observed with transfected cells and platelets, respectively, demonstrating that $P2Y_{12}$ protein is, indeed, expressed on the platelet surface.

When the chromosomal localization of the $P2Y_{12}$ gene was determined using the Stanford G3 panel [20] (Research Genetics), $P2Y_{12}$-specific primers mapped closest to STS-D13626, corresponding to the KIAA0001 gene recently identified as a UDP-glucose GPCR [21]. Both of these genes reside on chromosome 3q24-25, interval D3S1279-1280, a region that also includes the human $P2Y_1$ gene, (GeneMap 99, www.ncbi.nlm.nih.gov). Thus, this interval contains genes encoding at least three receptors, two of which ($P2Y_1$ and $P2Y_{12}$) mediate ADP-dependent platelet aggregation. Among GPCR's, $P2Y_{12}$ is most closely related to the UDP-glucose receptor[21](44% identical) but much less so to $P2Y_1$ (19% identical), suggesting that the UDP-glucose and $P2Y_{12}$ receptors are the product of a relatively recent gene duplication on chromosome 3.

UCSF LIBRARY

The predicted $hP2Y_{12}$ protein encodes four extracellular cysteines (see Fig 5). A critical role of cysteine residues in the function of the platelet ADP receptor has been suggested by the ability of thiol reagents to ablate ADP responses in platelets [1]. Indeed, the antithrombotic agent clopidogrel is proposed to inactivate the G_i-linked platelet ADP receptor through a mechanism in which it is metabolized to a sulphydryl species that modifies a cysteine residue on the receptor [22]. We found that brief exposure of oocytes expressing Kir3.1, 3.4 and $hP2Y_{12}$ to the thiol reagent p-chloromercuriphenylsulfonic acid (pCMBS) eliminated ADP-evoked current responses (Fig 2C). Inhibition was selective for the $P2Y_{12}$ receptor since activation of this signaling pathway by m2 muscarinic receptors expressed in the same oocytes was unaffected by treatment with pCMBS.

Nurden et al [10] have previously described a patient (ML) with a mild bleeding disorder. Platelets from ML exhibit impaired ADP-dependent platelet aggregation, greatly reduced ADP binding activity and lack the ability to inhibit cAMP levels in response to ADP. However, the $P2Y_1$-receptor mediated responses, such as intracellular calcium mobilization and shape change, are not affected, suggesting that this patient has a selective defect in the G_i-linked receptor. Analysis of PCR products from the $P2Y_{12}$ coding region from ML's genomic DNA revealed the presence of one mutant allele at this locus, as confirmed by direct sequencing of at least three independent PCR reactions. The mutation found in the $P2Y_{12}$ gene consists of a deletion of two nucleotides (TT<u>CA</u>TT) within the coding region, at amino acid 240 (near the amino-terminal

end of TM6), thus shifting the reading frame for 28 residues before introducing a premature stop codon (Fig. 5B). Biochemical studies suggest that platelets from ML lack G_i-linked ADP receptors, yet our sequence analysis indicates that this individual has one mutant and one wild-type $P2Y_{12}$ allele, at least so far as the protein coding region is concerned. This suggests one of two possibilities: the $P2Y_{12}$ mutation that we have identified exerts a dominant-negative effect, or ML harbors an additional mutation that eliminates expression of the allele containing a wild-type coding region. We evaluated the former possibility using our electrophysiological assay. First, no significant activity was observed when oocytes were injected with cRNA transcripts corresponding to the frame-shifted allele, demonstrating that this mutant is indeed non-functional. Moreover, when mutant and wild-type cRNA's were co-injected into oocytes at different ratios, no inhibition of the signal from the wild-type allele was observed, demonstrating that the mutant allele does not act in a dominant negative manner. We addressed the latter possibility by carrying out RT-PCR analysis with RNA from ML's platelets. Extremely low levels of $P2Y_{12}$–derived product were obtained compared to levels amplified from an unaffected individual or a control transcript encoding platelet GPIIB (figure 5C). In addition, sequence analysis of $P2Y_{12}$ RT-PCR products demonstrated that ML's $P2Y_{12}$ transcripts derive only from the mutant allele (i.e., no wild-type product was detected). We therefore conclude that ML's lack of functional G_i-coupled platelet ADP receptor activity is due to the fact that he only expresses the frame-shifted allele. Other patients with congenital platelet defects

UCSF LIBRARY

have been reported[23], and it will be interesting to determine whether these are also associated with mutations in $P2Y_{12}$.

In summary, we have characterized a novel cDNA from a platelet library that encodes the G_i-linked platelet ADP receptor. Genetic [4,5,10,23] and pharmacological [24,25] studies demonstrate that the G_i-linked receptor is critical for formation and stabilization of large platelet aggregates[26]. Additionally, the G_i-linked receptor is the target of the antithrombotic drugs clopidogrel and ticlopidine, which have been demonstrated to be efficacious in the treatment of a variety of thrombotic diseases (stroke, MI, peripheral vascular disease). However, these drugs work through a mechanism of covalent protein modification, which may underlie their recent association with the syndrome thrombotic thrombocytopenic purpura (TTP) [27], an immune-mediated response. Our studies demonstrate that the $P2Y_{12}$ receptor has a selective tissue distribution compared to other purinergic receptors (such as $P2Y_1$), making this receptor an extremely attractive target for the development of novel antithrombotics.

Methods

Platelet cDNA library Poly-A$^+$ mRNA from rat platelets was used to generate a directional oligo-dT primed cDNA library in the pcDNA3.1$^+$ vector. Approximately 320,000 clones were divided into 48 individual pools. Linearized cDNA templates from these pools were transcribed in vitro using T7 RNA polymerase (Ambion). Sib selection of a positive pool was performed to subfractionate the signal to the level of 96 clones. All were sequenced and a novel GPCR was further characterized. Rat $P2Y_{12}$ cDNA was used to isolate a human orthologue from a platelet I ZAP cDNA library. A full length $hP2Y_{12}$ cDNA expression construct was obtained by ligation of a I clone and a fragment derived by 3' RACE into the pcIneo expression vector (Promega). GenBank accession number for human $P2Y_{12}$ is XXXXX.

Platelet RT-PCR Whole blood (30 ml) was lysed and total RNA isolated using TriReagent BD (Molecular Research Center). First-strand cDNA was generated (Superscript 2, Life Technologies) and PCR (35 cycles) performed using the following mRNA-specific primers: The $P2Y_{12}$ 5' (5'-CCAGAATCAACAGTTATCA GGTAACC-3') and 3'(5'GTCAGTTAATATTTTTACTTAGCGCTTTGC-3') primers were annealed at 57°C, while the GPIIb 5' (5'-GTCAACGGGGATGGGAGGCATGA-3') and 3'(5'-GTCTGCCTCATCTCGAAGGAAGG-3') primers were annealed at 60°C . PCR products were analyzed by electrophoresis in 1% agarose and bands of the correct size were isolated for direct sequencing .

Electrophysiology Defolliculated *Xenopus laevis* oocytes were injected with a positive 500 clone pool (10 ng), $rP2Y_{12}$ (10 pg), $hP2Y_{12}$ (50 pg), Kir3.1, Kir3.4, PTX and hm2 (1 ng each) cRNAs as indicated. Three to seven days after injection, two-electrode voltage-clamp recordings were performed using a Geneclamp 500 amplifier (Axon Instruments) and a Maclab A/D converter (Maclab). Membrane potentials were clamped at –70 mV while the recording chamber was perfused at a rate of 2 ml/min with a solution containing (in mM) 70 KCl, 20 NaCl, 3 $MgCl_2$, 5 HEPES, pH 7.4, at room temperature. The KCl was replaced with NaCl to examine responses in zero potassium. Agonists and antagonists (Roche Molecular Biochemistry or Sigma) were diluted in the recording solution. Experiments using C1330-7 included 0.1 % dimethylsulfoxide to enhance its solubility in the perfusate.

Generation of stable mammalian cell lines and cAMP assays Chinese Hamster Ovary (CHO) cells or rat 2-9 fibroblasts, which are null for G_i-linked purinergic receptors, were transfected with $hP2Y_{12}$ or rP2Y12 cDNA's, respectively using FuGene reagent (Roche), and cells were cultured in the presence of G418 for 2 weeks to select for stable transfectants. For cAMP assays, stably transfected CHO cells expressing the $hP2Y_{12}$ plasmid were plated in 12-well dishes. Forty-eight hours later media was removed from the cells and replaced with serum-free media containing IBMX (0.25 μM final) and incubated at 37°C for 5 minutes. Cells were incubated for an additional 5 min with 10 μM forskolin, as well as the indicated agonists and antagonists. Pertussis toxin treatment (30 ng/ml) occurred for 20 hrs at 37°C prior to assay. Cyclic AMP

UCSF LIBRARY

levels were determined from aliquots of cell extracts in a radioimmunoassay (Amersham Biotrak cAMP ^{125}I assay system).

Northern and in situ hybridizations Northern blots of poly-A$^+$ RNA from human tissues (Clontech) or total human platelet RNA was hybridized with radiolabeled $hP2Y_{12}$ cDNA fragments under standard conditions. Digoxigenin-labeled *in situ* hybridization was performed on coronal rat brain sections using an RNA probe corresponding to the antisense sequence of $rP2Y_{12}$[28].

Flow cytometry Adult male Sprague-Dawley rats were anesthetized and whole blood isolated using citrate as anticoagulant. Platelet-rich plasma (PRP) was isolated by centrifugation and used for flow-cytometry analysis. A rabbit anti-sera (SynPep Corporation) was produced to the amino-terminal 23 residues of $rP2Y_{12}$. IgG was purified using protein-G sepharose. Rat PRP (2×10^6 cells) and cultured rat 2-9 fibroblasts transfected with $rP2Y_{12}$ cDNA (1×10^5 cells) were incubated with purified IgG (10-50 μg/ml) in FACS buffer (phosphate-buffered saline containing 0.1% BSA and 2% heat-inactivated fetal bovine serum) in a total volume of 100 μl for 1 hr at 4°C. Cells and platelets were then washed with cold FACS buffer and incubated with 2.5 μg/ml of FITC-conjugated goat anti-rabbit antibody for 30 min at 4°C. Cells and platelets were washed, resuspended in cold FACS buffer, and fluorescence of cell-bound secondary antibody was determined with a FACSort flow cytometer (Becton-Dickinson). Control samples contained cells without antibodies (for determination of autofluorescence), cells with control rabbit IgG, or secondary antibodies alone.

References

1. Mills, D. C. ADP receptors on platelets. *Thromb Haemost* 76, 835-56 (1996).
2. Jantzen, H. M. et al. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* 81, 111-7 (1999).
3. Daniel, J. L. et al. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* 273, 2024-9 (1998).
4. Leon, C. et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. *J Clin Invest* 104, 1731-7 (1999).
5. Fabre, J. E. et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in $P2Y_1$-deficient mice. *Nat Med* 5, 1199-202 (1999).
6. Gachet, C. et al. The thienopyridine ticlopidine selectively prevents the inhibitory effects of ADP but not of adrenaline on cAMP levels raised by stimulation of the adenylate cyclase of human platelets by PGE_1. *Biochem Pharmacol* 40, 2683-7 (1990).
7. Gachet, C. et al. ADP receptor induced activation of guanine nucleotide binding proteins in rat platelet membranes-an effect selectively blocked by the thienopyridine clopidogrel. *Thromb Haemost* 68, 79-83 (1992).

UCSF LIBRARY

8. Mills, D. C. B. et al. Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase. *Arterioscler. Thromb.* 12, 430-436 (1992).

9. Humphries, R. G., Tomlinson, W., Ingall, A. H., Cage, P. A. & Leff, P. A novel, highly potent and selective antagonist at human platelet P_{2T}-purinoreceptors. *Br. J. Pharmacol.* 113, 1057-1063 (1994).

10. Nurden, P. et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. *J. Clin. Invest.* 95, 1612-22 (1995).

11. Krapivinsky, G., Krapivinsky, L., Wickman, K. & Clapham, D. G bg binds directly to the G protein-gated K^+ channel, IKACh. *J. Biol. Chem.* 270, 29059-62 (1995).

12. Ohlmann, P. et al. The human platelet ADP receptor activates Gi2 proteins. *Biochem J* 312, 775-9 (1995).

13. MacFarlane, D. E., Srivastava, P. C. & Mills, D. C. B. 2-Methylthioadenosine[b-^{32}P]diphosphate:An agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets. *J. Clin. Invest.* 71, 420-428 (1983).

14. Hourani, S. M. O. & Hall, D. Receptors for ADP on human blood platelets. *Trends Pharmacol. Sci.* 15, 103-108 (1994).

15. Jantzen, H.-M. et al. Evidence for two distinct G protein-coupled ADP receptors mediating platelet activation. *Blood* 92, 303a (1998).

16. Boyer, J. L., Romero-Avila, T., Schachter, J. B. & Harden, T. K. Identification of competitive antagonists of the $P2Y_1$ receptor. *Mol Pharmacol* 50, 1323-9 (1996).

17. Palmer, R. K., Boyer, J. L., Schachter, J. B., Nicholas, R. A. & Harden, T. K. Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. *Mol Pharmacol* 54, 1118-23 (1998).

18. Filippov, A. K., Brown, D. A. & Barnard, E. A. The $P2Y_1$ receptor closes the N-type Ca(2+) channel in neurones, with both adenosine triphosphates and diphosphates as potent agonists. *Br J Pharmacol* 129, 1063-6 (2000).

19. Boyer, J. L., Lazarowski, E. R., Chen, X. H. & Harden, T. K. Identification of a P2Y-purinergic receptor that inhibits adenylyl cyclase. *J Pharmacol Exp Ther* 267, 1140-6 (1993).

20. Stewart, E. et al. An STS-based radiation hybrid map of the human genome. *Genome Res.* 7, 422-33 (1997).

21. Chambers, J. K. et al. A G protein-coupled receptor for UDP-glucose. *J Biol Chem* 275, 10767-71 (2000).

22. Savi, P. et al. Structure and activity of the active metabolite of Clopidogrel. *Thrombosis and Haemostasis* 82, 230 (1999).

23. Cattaneo, M. & Gachet, C. ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol* 19, 2281-5 (1999).

24. Jarvis, G. E., Humphries, R. G., Robertson, M. J. & Leff, P. ADP can induce aggregation of human platelets via both P2Y(1) and P(2T) receptors. *Br J Pharmacol* 129, 275-82 (2000).

UCSF LIBRARY

25. Hechler, B., Eckly, A., Ohlmann, P., Cazenave, J.-P. & Gachet, C. The $P2Y_1$ receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *Br. J. Haematology* 103, 858-866 (1998).

26. Humbert, M. et al. Ultrastructural studies of platelet aggregates from human subjects receiving clopidogrel and from a patient with an inherited defect of an ADP-dependent pathway of platelet activation. *Arterioscler Thromb Vasc Biol* 16, 1532-43 (1996).

27. Bennett, C. L. et al. Thrombotic thrombocytopenic purpura associated with Clopidogrel. *N. Eng. J. Med.* 325, 1371-2 (2000).

28. Caterina, M. J. et al. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816-824 (1997).

UCSF LIBRARY

Acknowledgements

We dedicate this paper to the memory of Ernie Peralta, for his friendship and valuable scientific insights. We thank H. Chuang for advice with electrophysiological assays, A. Laibelman and R. Scarborough for providing C1330-7, P. Castro and L. Komuves and members of the Baraban, Lowenstein and Basbaum laboratories for technical assistance and advice with the *in situ* hybridization. We thank E. Thompson, K. Simpson, S. Hollenbach, and K. Ministri-Madrid and the COR animal facility for excellent technical assistance, R.Wong and D. De Guzman for assistance with figures and C. Homcy, E. E. Reynolds, J. Topper and D. Phillips for helpful discussions. DJ is supported by funding from NIH and NIMH. GH is supported by a NIH predoctoral neuroscience training grant.

UCSF LIBRARY

Figure legends

Figure 1. $P2Y_{12}$ is a G protein–coupled receptor that responds to ADP. (A) Activation of potassium-dependent currents in *Xenopus* oocytes expressing $P2Y_{12}$ with Kir3.1 and 3.4. ADP (10 μM) was applied (short bars) in the presence or absence (long bar) of extracellular potassium (70 mM) while recording membrane currents in the whole-cell voltage clamp configuration. Oocytes injected with mRNA for Kir3.1 and 3.4 alone (top trace) do not exhibit significant currents in response to ADP application unless messages from a positive cDNA pool, the isolated rat $P2Y_{12}$ cRNA or the human $P2Y_{12}$ homologue are included (subsequent traces). (B) ADP-selective stimulation of potassium channel-dependent currents by $P2Y_{12}$ occurs via a pertussis toxin-sensitive pathway. UDP, adenosine (A), ATP-α-S or ADP (10 μM each) were sequentially applied to oocytes expressing the rat or human receptor with or without Kir3.1, 3.4 and pertussis toxin (PTX). (C) The agonist profile of $P2Y_{12}$ recapitulates that observed for the G_i-coupled platelet ADP receptor. Concentration-response curves for ADP and 2MeSADP are presented. Membrane currents were normalized in each oocyte to a response obtained with 10 μM ADP. Each point represents the mean values (+/- s.d.) from five independent oocytes. The Hill equation was used to fit the response data.

Figure 2. Currents stimulated by ADP in oocytes expressing $hP2Y_{12}$ with Kir3.1 and 3.4 are inhibited by 2MeSAMP, C1330-7 and a thiol reagent. (A) Current tracing showing reversible block of ADP (1 μM) responses by 2MeSAMP (10 μM)

and C1330-7 (1 μM), but not A3P5P (300 μM). The dot (.) indicates the start of a 15 sec application of ADP, while bars denote co-application with the antagonist. (B) 2MeSAMP and C1330-7 inhibition curves. Current responses were normalized to that elicited by ADP (500 nM) alone in each oocyte and plotted as the mean +/- s.d. Curves were fitted to the data using the Hill equation (n=5 independent oocytes for each point). (C) Selective ablation of $P2Y_{12}$ but not m2 muscarinic receptor signaling by the thiol reagent pCMBS. ADP (10 μM), carbachol (1 μM), and pCMBS (1 μM) were applied sequentially to an oocyte expressing both receptors concurrently with Kir3.1 and 3.4. Bars indicate periods of drug application (10 sec).

Figure 3. Activation of $hP2Y_{12}$ in CHO cells inhibits adenylyl cyclase. (A) Receptor coupling to adenylyl cyclase was assessed as ADP-mediated (0.1-10 μM) inhibition of forskolin-stimulated (10 μM) cAMP accumulation (CON=control, normalized to 100%). (B) The effect of the specific antagonists 2MeSAMP (2-MES)(50 μM) and C1330-7 (50 μM) on repression of ADP-mediated (1 μM) forskolin-stimulated cAMP levels. (C) Effect of pertussis toxin (PTX) pretreatment on the inhibition by 10 μM ADP of forskolin-stimulated cAMP levels. Results are the mean +/- s.d. of three representative experiments performed in triplicate.

Figure 4. $P2Y_{12}$ receptor is selectively expressed in platelets and brain. (A, B) Northern analysis of $hP2Y_{12}$ transcripts. All lanes contain 2 μg poly-A^+ mRNA except samples from platelet and Jurkat cells (20 μg each). (C) $rP2Y_{12}$

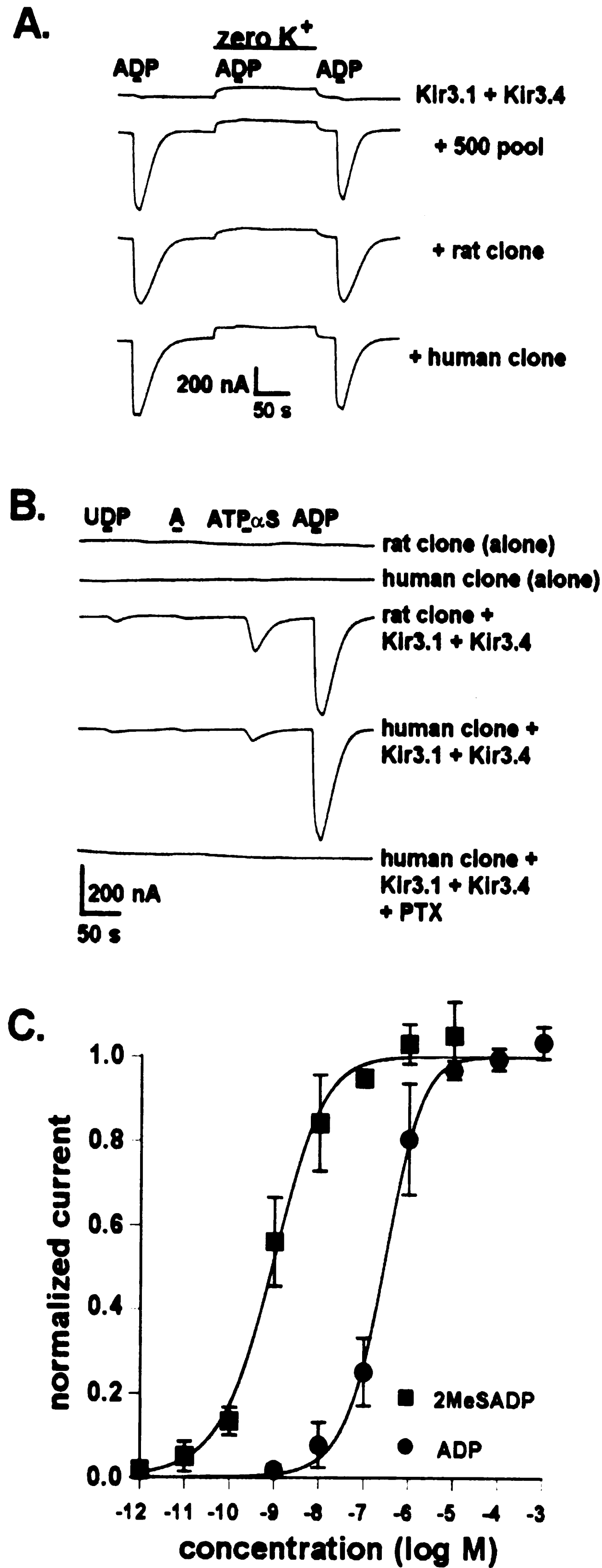
UCSF LIBRARY

transcripts are distributed throughout the brain in presumptive glia. Staining was equally abundant in fiber tracts (corpus callosum, cc) and regions enriched for neuronal cell bodies (dentate gyrus, dg; arcuate nucleus of the hypothalamus, an), but absent from vasculature (pericallosal artery, pa). Control (sense) riboprobes did not stain these regions. Ventricular structures are also indicated (dorsal third ventricle, d3v; third ventricle, 3v). (D) FACS analysis of rat platelets stained with $rP2Y_{12}$ antisera (filled peak) or a control IgG (unfilled peak). (E) FACS analysis of rat 2-9 fibroblasts transfected with the $rP2Y_{12}$ cDNA clone (filled peak) or untransfected rat 2-9 fibroblasts (unfilled peak).

Figure 5. A frame-shift mutation within the $hP2Y_{12}$ gene is associated with a bleeding disorder. (A) Deduced amino acid sequence of the $hP2Y_{12}$ protein and alignment with other homologous receptor sequences. The putative membrane-spanning domains are designated with bars above the sequence. $hP2Y_{12}$ sequence is aligned with the sequences of $hP2Y_1$ receptor (also expressed in platelets and activated by ADP), as well as with the human UDP-glucose receptor, with which it shares greatest homology. Shading denotes amino acid identity (black) or similarity (gray); asterisks (*) denote extracellular cysteine residues. (B) A $P2Y_{12}$ allele from a patient (ML) with defective ADP-dependent aggregation contains a 2 base pair deletion, resulting in a frame-shift mutation and a premature truncation of the protein. No such mutation was observed in PCR products amplified and sequenced from genomic DNA of twenty randomly chosen individuals. (C) Patient ML has abnormally low levels of RT-PCR product

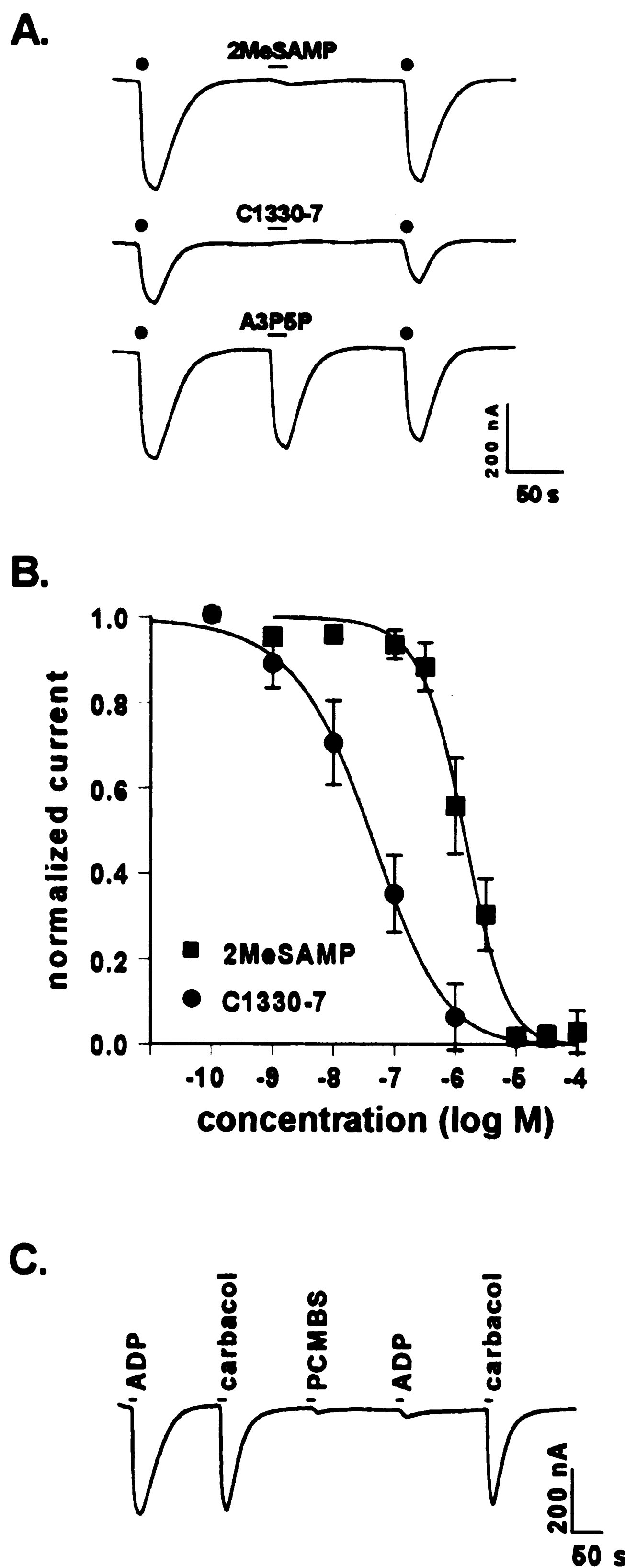
derived from $P2Y_{12}$ mRNA. RT-PCR using either $P2Y_{12}$- (lanes 1-4) or GPIIb- (lanes 5-8) specific primers was performed using whole blood RNA from patient ML (lanes 1,2,5,6) or a control (CON) sample (lanes 3,4,7,8). PCR reactions performed on RNA samples not reverse transcribed control for genomic DNA contamination (lanes 1,3,5,7). A 1.1 kb product encoding the $P2Y_{12}$ ORF was amplified from the control sample, but virtually absent from ML (a faint product can be observed upon longer exposure). In contrast, the amount of product (0.77 kb) amplified from GPIIb mRNA was equivalent between ML and control. Sequence analysis reveals that ML's $P2Y_{12}$ RT-PCR product derived solely from the mutant allele.

Figure 1



UCSF LIBRARY

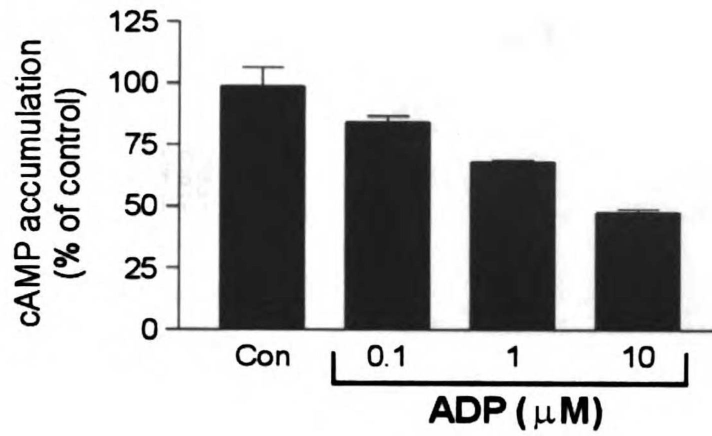
Figure 2



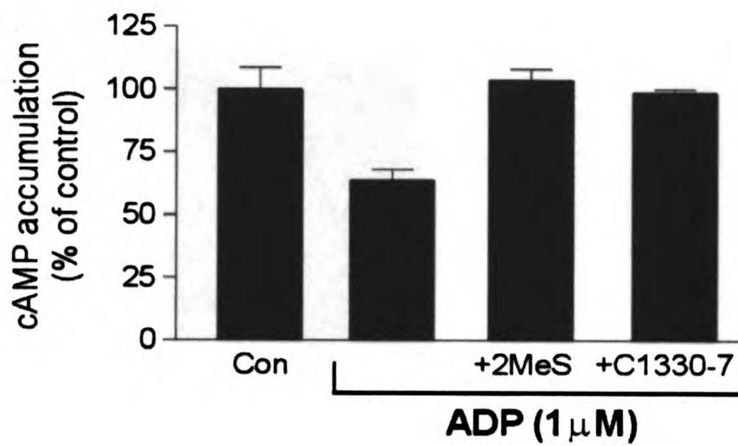
UCSF LIBRARY

Figure 3

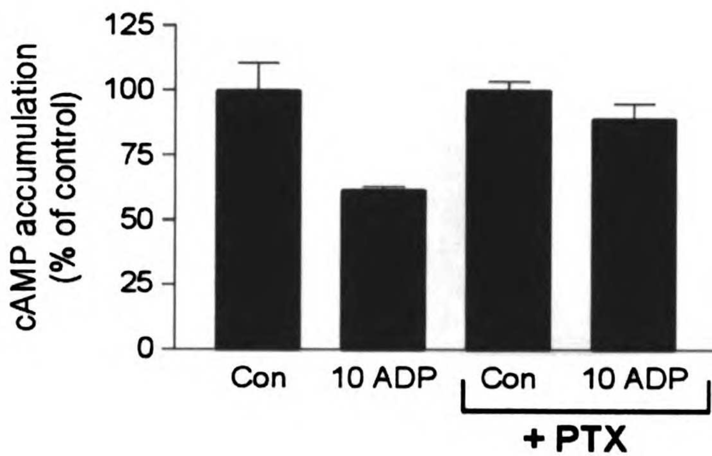
A.



B.

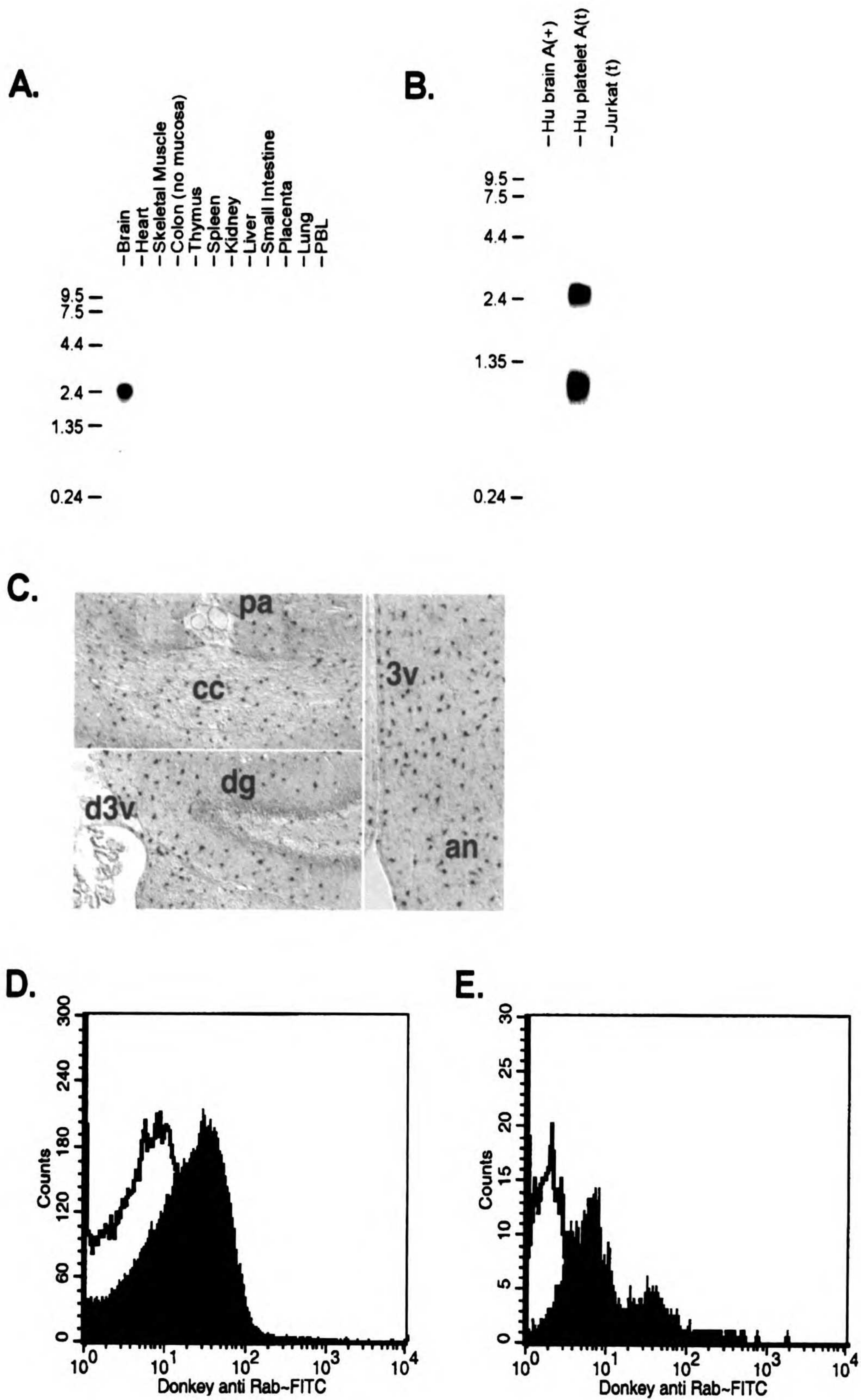


C.



UCSF LIBRARY

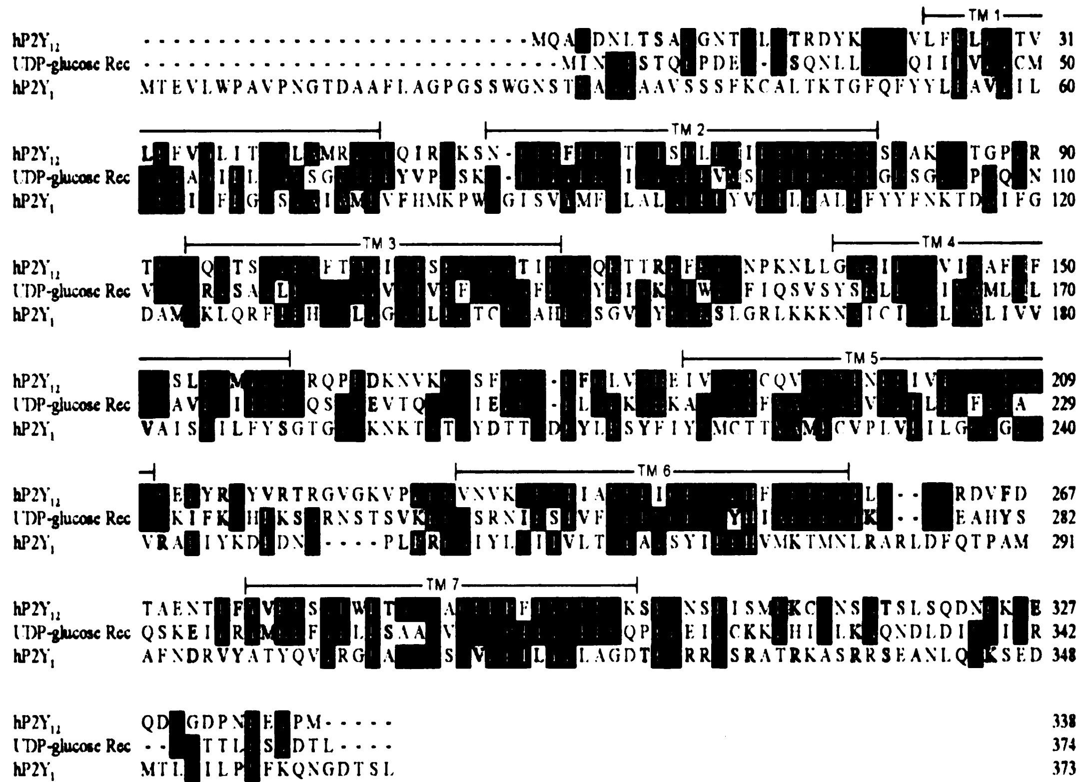
Figure 4



UCSF LIBRARY

Figure 5

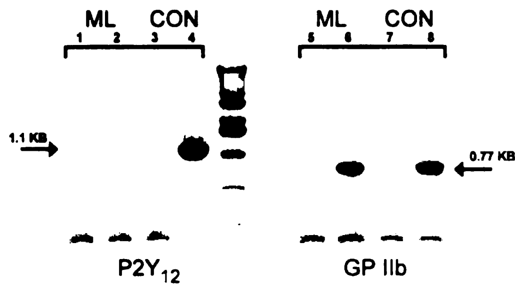
A.



B.

Wild type aa	246	V	K	V	F	I	I	I	A	V	F	F
Wild type nucleotide		GTCAAAGTTTTCATTATCATTGCTGTATTCTTT										
Mutant nucleotide		GTCAAAGTTTT- TTATCATTGCTGTATTCTT										
Mutant aa	246	V	K	V	F		Y	H	C	C	I	L

C.



CHAPTER 3

The $P2Y_{12}$ Receptor Regulates Microglial Activation by Extracellular Nucleotides

The P2Y$_{12}$ Receptor Regulates Microglial Activation by Extracellular Nucleotides

Sharon Haynes*, Gunther Hollopeter*, Guang Yang†, Wen-Biao Gan†, and David Julius*‡

* *Department of Cellular and Molecular Pharmacology*

University of California, San Francisco, CA 94143-2140

† *Molecular Neurobiology Program, Skirball Institute, New York University School of Medicine, New York, NY 10016*

‡To whom correspondence should be addressed:

telephone: 415-476-0431

telefax: 415-502-8644

email: julius@cmp.ucsf.edu

UCSF LIBRARY

Microglia are the primary immune sentinels of the central nervous system. Following injury or infection, these cells migrate or extend processes towards sites of tissue damage [1-3], where they clear debris and release nitric oxide, cytokines, and other neurotoxic or neurotrophic agents [4-8]. As such, microglial activation, or lack thereof, has been proposed to influence degenerative and regenerative processes in the brain and spinal cord. CNS injury can be accompanied by the release of nucleotides [9], which have been proposed to serve as signals for microglial activation or chemotaxis [2,10,11]. Microglia express both ionotropic (P2X) and metabotropic (P2Y) purinoceptors [12], including a G_i-coupled subtype that has been implicated in ATP/ADP-mediated enhancement of microglial migration *in vitro* [11]. Here we show that microglia from mice lacking the G_i-coupled $P2Y_{12}$ receptor are morphologically unresponsive to ATP *in vitro* or *in vivo*. $P2Y_{12}^{-/-}$ mice also show a dramatic reduction in microglial response to cortical tissue damage. Moreover, $P2Y_{12}$ expression is robust in the resting state, but dramatically reduced after microglial activation. These results suggest that $P2Y_{12}$ is a primary site at which nucleotides act to induce morphological changes of microglia at early stages of the response to local CNS injury.

In the brain, microglia project processes towards sites of mechanical injury or laser ablation [2,3]. These responses are mimicked by injection of ATP and attenuated by broad-spectrum inhibitors of purinergic signalling [2], focusing attention on the role of nucleotides as regulators of microglial function *in vivo*. Microglia express an array of ionotropic ($P2X_4$ and $P2X_7$) and metabotropic ($P2Y_1$, $P2Y_2$, and $P2Y_{12}$) purinergic receptors that have been proposed to play important roles in microglial activation, movement, or paracrine signalling [12,13]. ATP or ADP-evoked membrane ruffling and chemotaxis of cultured microglia have been shown to occur through a pertussis toxin-sensitive signalling pathway [11]. The vast majority of metabotropic P2Y receptors transduce their signals through G_q/phospholipase C pathways [14]. In this regard, the $P2Y_{12}$ subtype is unique in that it couples to G_i/adenylyl cyclase [15,16], making it an attractive candidate for mediating morphological responses of microglia to nucleotides. The paucity of subtype-selective agonists or antagonists has hindered efforts to rigorously examine physiological roles for specific P2 receptor subtypes *in vivo*, and we have therefore taken a genetic approach to test the hypothesis that a $P2Y_{12}$-like receptor contributes to injury-evoked activation of microglia in the nervous system.

We, and others, have previously shown that $P2Y_{12}$ transcripts are expressed in glial cells of the spinal cord and brain [13,15]. To determine whether receptor protein is located on the microglial cell surface, we generated an antibody that recognizes the C-terminus of mouse $P2Y_{12}$ and used this polyclonal antiserum to stain sections from spinal cord and brain. Indeed, robust signals

were clearly observed in a population of glial cells evenly distributed throughout both white and gray matter. Identification of these cells as microglia was confirmed by co-localization with two independent microglial markers [17], including enhanced green fluorescent protein (GFP) expressed from the CX_3CR1 fractalkine receptor promoter (Fig. 1a), and the integrin CD11b (data not shown). Moreover, $P2Y_{12}$ immunoreactivity was completely absent from astrocytes. Higher magnification images clearly showed that $P2Y_{12}$ protein was localized exclusively to the cell surface, including the elaborate ramified processes emanating from the cell body (Fig. 1b). Thus $P2Y_{12}$ is poised to enable microglia to detect changes in extracellular nucleotide concentration as they survey their local environment. Importantly, $P2Y_{12}$-deficient mice were devoid of $P2Y_{12}$ immunoreactivity (Fig. 1a), but showed normal prevalence, distribution, and morphology of resting microglia (Supplemental Fig. 1), suggesting that the receptor is not required for proper development or CNS localization of these cells.

Microglia are believed to mediate a number of physiological responses to neural injury that include both short and long term actions [6]. Indeed, the expression of various cell surface markers and cellular phenotypes are known to change following CNS injury [18]. We therefore asked whether a change in $P2Y_{12}$ expression accompanies microglial activation. To address this question, we examined $P2Y_{12}$ immunoreactivity in response to a bolus injection of lipopolysaccharide (LPS) into the striatum, a treatment that activates microglia within the injected area [19] and has been shown to decrease 2MeSADP-evoked

UCSF LIBRARY

calcium increases in cultured mouse microglia [20]. We found that virtually all microglia within proximity of the injection site exhibited an amoeboid morphology and completely lacked $P2Y_{12}$ immunoreactivity (Fig. 2a). Moreover, the rare cell that did express $P2Y_{12}$ antigen was characterized by a highly ramified, resting morphology (data not shown). Reduced $P2Y_{12}$ expression was confirmed at the transcriptional level by *in situ* hybridization histochemistry and northern blotting, where a substantial decrease in messenger RNA levels was observed (Fig. 2b, c). This was further validated by quantitative real-time RT-PCR performed with total RNA isolated from a region of brain containing the injection site, which showed a 60% reduction in $P2Y_{12}$ transcripts. Taken together, our data support an inverse correlation between microglial activation and $P2Y_{12}$ expression, and suggest that $P2Y_{12}$ is an excellent molecular marker for visualizing microglia in the resting state. These findings also indicate that $P2Y_{12}$ is likely to participate in early stages of microglial response to injury, when they switch from an inactive to activated state.

In light of these observations and proposed roles for nucleotides in regulating microglial motility, we sought to examine phenotypes in $P2Y_{12}$–deficient mice using experimental paradigms that monitor cellular responses within minutes of stimulus application. First, we used an *in vitro* culture system to address the potential involvement of $P2Y_{12}$ in ATP/ADP-mediated actin-based lamelopodial extension. When microglia are isolated from neonatal rodent brain and subjected to serum starvation for several hours, they revert to a presumptive resting or inactivated state based on morphological and

immunological criteria [21]. Under these conditions, the vast majority (~87%) of microglia from wild-type mice displayed robust membrane ruffling upon application of ADP or ATP (50 μM) (Fig 3), consistent with previous observations [11]. In contrast, cells from $P2Y_{12}^{-/-}$ mice showed no response above that elicited by UTP (50 μM) or vehicle alone (~7%). Application of macrophage colony stimulating factor (mCSF, 200 ng/ml) produced equivalent responses in wild-type and $P2Y_{12}$-deficient microglia (~74%), demonstrating that the absence of $P2Y_{12}$ does not generally disrupt signalling mechanisms required for actin polymerization (Fig. 3).

To examine directed motility in response to a localized nucleotide source, we placed purified microglia in a Dunn chemotaxis chamber and observed their behavior in a gradient of ADP or ATP (50 → 0 μM) using phase contrast time-lapse microscopy. Microglia from wild-type mice showed a clear and robust polarization or chemotaxis toward the nucleotide source, whereas $P2Y_{12}$-deficient microglia showed no evidence of membrane ruffling, polarization, or directed movement (Fig 3c, d).

These results demonstrate that the $P2Y_{12}$ receptor is essential for directed movement of microglia towards extracellular nucleotides *in vitro*. Is this relevant to the mechanism whereby nucleotides or tissue injury promote rapid microglial responses *in vivo*? To address this question, we injected ATP (20 mM) into the neocortex of $P2Y_{12}^{+/+}CX_3CR1^{+/GFP}$ or $P2Y_{12}^{-/-}CX_3CR1^{+/GFP}$ mice and followed changes in microglial morphology using two-photon time-lapse microscopy to visualize GFP fluorescence. As previously described [2], microglia in wild-type

animals showed very active extension of cellular processes toward the site of nucleotide injection (Fig. 4a, b). In contrast, $P2Y_{12}$-deficient mice showed greatly reduced responses during an equivalent 40 min post-injection period, demonstrating that the $P2Y_{12}$ receptor is essential for ATP-mediated process extension of microglia *in vivo*.

Next, we asked whether $P2Y_{12}$ is also required for the ability of microglia to rapidly detect and respond to tissue injury, such as that inflicted by focal laser ablation. As observed with ATP injection, microglia from wild-type mice showed immediate and robust responses characterized by the formation of projections toward the site of damage (Fig. 4c, d). Here again, $P2Y_{12}^{-/-}$ mice showed a dramatically reduced chemotaxic response over an equivalent 40 min observation period. Interestingly, when microglia from mutant mice were examined at 2 hours post-injury, the degree of process extension into the damaged area approached that observed with wild-type controls at the 32 min time point, demonstrating that lack of $P2Y_{12}$ receptors significantly delays, but does not abolish the ability of microglia to respond to tissue damage. Finally, recent imaging studies have shown that even in the absence of an injury stimulus, microglia exhibit appreciable, but non-directed process extension, possibly representing homeostatic surveillance of their environment [2,3]. We found that over 10 minutes, the average absolute length change of randomly analyzed processes from wild-type or P2Y12-deficient microglia were not significantly different (n=31-36 processes from 3 animals per genotype; $P > 0.16$), suggesting that the receptor is involved primarily in mediating responses to cellular injury.

Recently, it has been shown that ATP is an important signalling molecule in the response of microglia to laser induced damage in the CNS [2]. We now provide genetic proof that nucleotides report tissue injury by activating $P2Y_{12}$, a G_i-coupled purinergic receptor on microglia. In addition to $P2Y_{12}$, recent studies have identified a second ADP-activated G_i-coupled receptor, $P2Y_{13}$, with similar pharmacological properties [22]. While we can detect low levels of $P2Y_{13}$ transcript in microglia, we were unable to observe protein expression using a $P2Y_{13}$–specific antibody (Supplemental Fig. 2). These results, together with our functional studies, suggest that $P2Y_{12}$ is the primary site through which nucleotides mediate the rapid microglial responses that we describe here, although a contribution by $P2Y_{13}$ cannot be formally excluded.

The $P2Y_{12}$ receptor was initially identified on platelets, where it regulates their conversion from an inactive to active state during the clotting process [15,23,24]. Our data suggest that $P2Y_{12}$ receptors also function early in the microglial activation process to initiate what has recently been described as a switch from a state of basal, undirected motility to one in which cellular ramifications show rapid and highly directed extension towards sites of tissue damage [2,3]. Microglia express a plethora of purinergic receptor subtypes, suggesting that nucleotides play numerous roles in modulating immune surveillance of the CNS in response to a range of pathophysiological insults. For example, pharmacological studies have implicated P2X receptors on microglia in the production of neuropathic pain [25] or the release of neuroprotective or proinflammatory agents, such as tumor necrosis factor-a and interleukin-1β [26,27]. The further development of selective

UCSF LIBRARY

P2 receptor drugs and genetic models will help to elucidate both neuroprotective and neurodegenerative roles for these enigmatic immune effector cells of the spinal cord and brain.

Methods

Mouse strains $P2Y_{12}^{-/-}$ mice [24] were mated with $CX_3CR1^{+/GFP}$ animals [17] to generate paired $P2Y_{12}^{+/+}$ $CX_3CR1^{+/GFP}$ and $P2Y_{12}^{-/-}CX_3CR1^{+/GFP}$ littermates for these studies.

Immunohistochemistry and in situ hybridization Anti-$P2Y_{12}$ receptor polyclonal antibody was generated by immunizing rabbit with a synthetic peptide corresponding to the mouse $P2Y_{12}$ C-terminus (NH_2-Cys-Gly-Thr-Asn-Lys-Lys-Lys-Gly-Gln-Glu-Gly-Gly-Glu-Pro-Ser-Glu-Glu-Thr-Pro-Met-OH; Anaspec, Inc). Antibody was affinity purified using a Sulfolink coupling gel (Pierce) to immobilize the antigenic peptide. This antibody showed immunoreactivity for mouse, rat, and human $P2Y_{12}$ as assessed by immunofluorescence and western blotting of transfected HEK293T cells expressing each of these receptors. Brain tissue was fixed and sectioned (30 μm thick) on a freezing cryotome, blocked for 30 min in 10% normal goat serum/0.1% triton in PBS and stained with $P2Y_{12}$, CD11b (eBiosciences), or GFAP (Pharmingen) primary antibodies overnight at 4°C, washed and visualized with goat anti-rabbit Alexa 564 or 594, goat anti-rat Alexa 594, or goat anti-mouse Alexa 635 secondary antibodies (Molecular Probes), respectively. Images were taken on a Zeiss Pascal or LSM 510 Meta confocal microscope using either a 40x oil- or 20x multi-immersion objective.

Primary microglia cell culture, membrane ruffling, and chemotaxis assays

Primary microglia were isolated from P1-P3 mice using a modified version of the mixed glial culture technique as previously described [28]. Briefly, brains were dissected, homogenized by passing through an 18G needle, and cultured in DME-H21 with 10% heat-inactivated FBS and penicillin/streptamycin. After 12-

14 days, cultures were gently shaken by hand for 15 min and microglia collected as floaters, resulting in >99% purity as assessed by GFP expression. Isolated microglia were spotted onto either glass chamber slides (Fisher) for membrane ruffling assays or fibronectin coated coverslips (Becton Dickinson) for chemotaxis assays, washed, and incubated for 4 hours in serum-free DME-H21.

Membrane ruffling assays were performed as described [11] by replacing DME-H21 with fresh DME-H21 containing no stimulus (negative control) or ADP, ATP, or UTP (50 μM), 2-MeSADP (0.5 μM, Sigma), or mCSF (200 ng/ml, BD Biosciences). Microglia were incubated for 5 min at 37°C, fixed with 4% PFA, and stained with rhodamine-phalliodin (Molecular Probes). Chemotaxis assays were performed using the Dunn chamber [29]. Cells were incubated in DME-H21 and ADP or ATP (50 μM) was applied to one side of the cells. Distance and direction of movement by the cell's leading edge was monitored over a 30 min period by phase contrast time-lapse microscopy; the average distance migrated in the absence of a stimulus (<0.8 mm) was subtracted to obtain final values. Images were processed and analyzed using the National Institutes of Health ImageJ software.

***in vivo* imaging of microglia** GFP expressing microglia were imaged by two-photon time-lapse microscopy as described [2]. Briefly, ATP (20 mM) was diluted in artificial CSF for intracranial injections. A z-stack (~100 μm in depth, 2 μm z-steps) was acquired every 4 min for 40 min. A projection of 15 z-steps centered on the laser ablation or injection site was constructed for each time point. To quantify microglial response to ATP injection, we monitored changes in GFP

UCSF LIBRARY

fluorescence as processes entered a circular zone of 70 μm in diameter centered on the injection needle, normalized to an outer region 136 μm in diameter. Quantification of microglial response to laser ablation was performed similarly using a circular zone of 35 μm in diameter centered on the ablation, normalized to an outer region of 97 μm in diameter.

LPS injections and analysis of $P2Y_{12}$ expression Mice were anesthetized with ketamine/zylazine and placed in a stereotaxic apparatus. A small incision was made in the scalp and a small hole drilled through the skull over the area of injection. The needle was positioned at 1 mm anterior, 1.5 mm lateral, and 3 mm ventral to bregma and 1 μl of 5 mg/ml LPS (Sigma) or PBS (control) was bilaterally injected into the striatum. The wound was closed and mice allowed to recover for 4 days prior to tissue collection. For immuno- and *in situ* hybridization histochemistry, PFA-fixed (20 – 30 mm thick) brain sections were incubated with anti-$P2Y_{12}$ antibody, or a digoxygenin-labeled cRNA probe containing the entire mouse $P2Y_{12}$ coding region (SacI-NsiI fragment). For *in situ* hybridization experiments, GFP expression was visualized with an anti-GFP antibody (Molecular Probes). For northern blot and RT-PCR analyses, mice were perfused with PBS and RNA was extracted from a coronal brain slab that included a region 2 mm posterior and anterior to the injection site. Poly A+ RNA was purified with a micro-FastTrack kit (InVitrogen) and analyzed as previously described [30] using a ^{32}P-labeled BbvCI cDNA fragment containing the $P2Y_{12}$ coding region. Real-time RT-PCR was performed on first-strand cDNA generated from DNAse-treated total RNA using the following primer pairs for

$P2Y_{12}$: 5'-CATTGCTGTACACCGTCCTG-3' and

5'-GGCTCCCAGTTTAGCATCAC-3'. Analysis was carried out as previously described [31].

References

1. Stence, N., Waite, M. & Dailey, M. E. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* **33**, 256-66 (2001).
2. Davalos, D. et al. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* **8**, 752-8 (2005).
3. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314-8 (2005).
4. Chao, C. C., Hu, S., Molitor, T. W., Shaskan, E. G. & Peterson, P. K. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol* **149**, 2736-41 (1992).
5. Kreutzberg, G. W. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* **19**, 312-8 (1996).
6. Streit, W. J. in *Microglia in the regenerating and degenerating central nervous system* (ed. Streit, W. J.) 1-14 (Springer, New York, 2002).
7. Hanisch, U.-K. in *Microglia in the regenerating and degenerating central nervous system* (ed. Streit, W. J.) 79-124 (Springer, New York, 2002).
8. Zhang, C. & Tso, M. O. Characterization of activated retinal microglia following optic axotomy. *J Neurosci Res* **73**, 840-5 (2003).
9. Wang, X. et al. P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med* **10**, 821-7 (2004).

References

1. Stence, N., Waite, M. & Dailey, M. E. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* **33**, 256-66 (2001).
2. Davalos, D. et al. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* **8**, 752-8 (2005).
3. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314-8 (2005).
4. Chao, C. C., Hu, S., Molitor, T. W., Shaskan, E. G. & Peterson, P. K. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol* **149**, 2736-41 (1992).
5. Kreutzberg, G. W. Microglia: a sensor for pathological [illegible] *Trends Neurosci* **19**, 312-8 (1996).
6. Streit, W. J. in *Microglia in the regenerating and* [illegible] *nervous system* (ed. Streit, W. J.) 1-14 (Springer, [illegible]).
7. Hanisch, U.-K. in *Microglia in the regenera*[illegible] *nervous system* (ed. Streit, W. J.) 79-124 [illegible]
8. Zhang, C. & Tso, M. O. Characteriz[illegible] n. following optic axotomy. *J* [illegible]
9. Wang, X. [illegible] y of cord [illegible]

10. Neary, J. T., Rathbone, M. P., Cattabeni, F., Abbracchio, M. P. & Burnstock, G. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci* **19**, 13-8 (1996).

11. Honda, S. et al. Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. *J Neurosci* **21**, 1975-82 (2001).

12. Inoue, K. Microglial activation by purines and pyrimidines. *Glia* **40**, 156-63 (2002).

13. Sasaki, Y. et al. Selective expression of Gi/o-coupled ATP receptor P2Y12 in microglia in rat brain. *Glia* **44**, 242-50 (2003).

14. Burnstock, G. in *P2 purinoceptors: localization, funciton, and transduction mechanisms* (eds. Chadwick, D. & Goode, J.) 1-34 (John Wiley & Sons, New York, 1996).

15. Hollopeter, G. et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**, 202-7 (2001).

16. Zhang, F. L. et al. ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. *J Biol Chem* **276**, 8608-15 (2001).

17. Jung, S. et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol* **20**, 4106-14 (2000).

18. Streit, W. J., Graeber, M. B. & Kreutzberg, G. W. Functional plasticity of microglia: a review. *Glia* **1**, 301-7 (1988).

19. Lee, J. C. et al. Accelerated cerebral ischemic injury by activated macrophages/microglia after lipopolysaccharide microinjection into rat corpus callosum. *Glia* **50**, 168-81 (2005).

20. Moller, T., Kann, O., Verkhratsky, A. & Kettenmann, H. Activation of mouse microglial cells affects P2 receptor signaling. *Brain Res* **853**, 49-59 (2000).

21. Salimi, K. & Humpel, C. Down-regulation of complement receptor 3 and major histocompatibility complex I and II antigen-like immunoreactivity accompanies ramification in isolated rat microglia. *Brain Res* **946**, 283-9 (2002).

22. Communi, D. et al. Identification of a novel human ADP receptor coupled to G(i). *J Biol Chem* **276**, 41479-85 (2001).

23. Foster, C. J. et al. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest* **107**, 1591-8 (2001).

24. Andre, P. et al. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest* **112**, 398-406 (2003).

25. Tsuda, M. et al. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* **424**, 778-83 (2003).

26. Suzuki, T. et al. Production and release of neuroprotective tumor necrosis factor by P2X7 receptor-activated microglia. *J Neurosci* **24**, 1-7 (2004).

27. Chakfe, Y. et al. ADP and AMP induce interleukin-1beta release from microglial cells through activation of ATP-primed P2X7 receptor channels. *J Neurosci* **22**, 3061-9 (2002).

28. Giulian, D. & Baker, T. J. Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci* **6**, 2163-78 (1986).

29. Allen, W. E., Zicha, D., Ridley, A. J. & Jones, G. E. A role for Cdc42 in macrophage chemotaxis. *J Cell Biol* **141**, 1147-57 (1998).

30. Caterina, M. J. et al. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816-24 (1997).

31. Luecke, H. F. & Yamamoto, K. R. The glucocorticoid receptor blocks P-TEFb recruitment by NFkappaB to effect promoter-specific transcriptional repression. *Genes Dev* **19**, 1116-27 (2005).

Acknowledgements

We are grateful to H. Morisaki, J. Braz, and A. So for experimental advice and assistance, and D. Littman for providing CX3CR1$^{+/GFP}$ mice. This work was supported by a NIH predoctoral fellowships from NIGMS (S.H.) and the American Heart Association (G.H.), and by the Christopher Reeve Paralysis Foundation and the NIMH Silvio Conte Center for Neuroscience Research at UCSF (D.J.).

Figure Legends

Figure 1. $P2Y_{12}$ immunoreactivity is localized to the cell membrane of microglia in the brain. **a**, $P2Y_{12}$ antibody (red) was used to visualize receptors in brain sections from wild-type (+/+) and $P2Y_{12}$-deficient (-/-) mice. $P2Y_{12}$ immunoreactivity co-localized with GFP (green), which was expressed under control of the CX3CR1 promotor to specifically label microglia. $P2Y_{12}$ staining was excluded from astrocytes expressing GFAP (blue). Scale bar = 20 mm. **b**, Higher magnification shows that $P2Y_{12}$ (red) is localized to the cell membrane, whereas soluble GFP (green) allows for visualization of the cell body and cytoplasm. Scale bar = 5 mm.

Figure 2. $P2Y_{12}$ expression is diminished in activated microglia. **a**, Brain sections from vehicle (PBS)- or lipopolysaccharide (LPS, 5 mg)-injected $P2Y_{12}{}^{+/+}CX_3CR1^{+/GFP}$ mice were examined for $P2Y_{12}$ immunoreactivity (red). Note the absence of $P2Y_{12}$ staining 4 days after LPS injection. Similar results were obtained in 5 independent experiments. Scale bar = 20 mm. **b**, *In situ* hybridization with a $P2Y_{12}$ antisense probe also showed a substantial loss of $P2Y_{12}$ expression following LPS injection. A GFP antibody was used to visualize microglia (green). Scale bar = 20 mm. **c**, Northern blot analysis of poly A+ brain RNA (2 mg) from a region surrounding the injection site showed a decrease in $P2Y_{12}$ expression compared to PBS-injected control. Probe specificity was verified by lack of signal from $P2Y_{12}{}^{-/-}$ tissue. Cyclophilin transcripts were analyzed to verify equivalent sample loading. **d**, Quantitative real-time RT-PCR

of samples analyzed by northern blot analysis confirmed the observed decrease in $P2Y_{12}$ transcript levels after injection of LPS. Signals were normalized to those obtained with ribosomal protein L19-specific primers (RPL19).

Figure 3. Nucleotide-evoked membrane ruffling and chemotaxis are abolished in microglia lacking the $P2Y_{12}$ receptor. **a**, Brain microglia from wild-type (+/+) and $P2Y_{12}$-deficient (-/-) mice were exposed to vehicle (DMEH21), ADP (50uM) or macrophage-colony stimulating factor (mCSF, 200ng/ml). Membrane ruffling was visualized within 5 min. by staining with rhodamine-phalloidin (red) to detect actin polymerization (white arrows). Scale bar = 20 mm. **b**, Fraction of microglia undergoing membrane ruffling in the presence of vehicle (DMEH21), UTP (50 mM), ADP (50 mM), ATP (50 mM) 2MeSADP (5 mM), and mCSF (200 ng/ml). Asterisks indicate $P < 0.001$; $n \geq 9$ trials per genotype for each experimental condition; Student's t-test. Values represent mean ± s.e.m. **c**, Microglia from wild-type and $P2Y_{12}$ mutant mice were placed in a gradient of ATP (50 → 0 mM) using a Dunn chemotaxis chamber and visualized phase contrast time-lapse microscopy. Microglia from wild-type mice showed robust chemotaxis toward the nucleotide source (white arrows indicate leading edge of migrating cell at 5 and 30 min time points), whereas $P2Y_{12}$-deficient microglia showed a dramatically decreased response. Scale bar = 20 mm. **d**, Scatter plot indicates distance (mm) traveled by the leading edge of wild-type (grey) or mutant (black) microglia in a gradient of ADP or ATP after 30 min. Values are plotted in the x-y directions relative to location of the nucleotide source.

Figure 4. Loss of $P2Y_{12}$ receptors abrogates response of microglia to ATP injection or focal laser ablation in the living brain. **a**, Microglia from wild-type (+/+) or $P2Y_{12}$-deficient (-/-) GFP-expressing mice were visualized *in vivo* using two-photon time-lapse microscopy. Exuberant process extension towards a point source of ATP (20 mM from injection needle, red) was observed in wild-type, but not mutant mice. Scale bar = 20 mm. **b**, Quantification of ATP-evoked process extension over a 40 min time course. $P2Y_{12}^{+/+}$ is shown in grey and $P2Y_{12}^{-/-}$ in black (n = 4 mice per genotype). **c**, Tissue ablation by the two-photon laser (white circle in center) results in microglial process extension towards the site of injury. Microglia from mutant mice showed greatly reduced responses over a 40 min observation period. Scale bar = 20 mm. **d**, Quantification of process extension toward the site of laser ablation (n = 4 mice per genotype). $P2Y_{12}$ mutants were analyzed at 2 hrs post-injury (n = 3). For panels **b** and **d**, *P< 0.05, **P<0.01, ***P<0.001; Student's t-test. Values represent mean ± s.e.m.

Supplemental Fig. 1 Microglia from $P2Y_{12}$-deficient mice have normal morphology and prevalence within the CNS. **a**, Microglia were quantified by counting GFP-positive cell bodies in spinal cord sections from wild-type (+/+) or $P2Y_{12}$-deficient (-/-) $CX_3CR1^{+/GFP}$ mice. **b**, Similar quantification was performed from paraformaldehyde-fixed whole-mount retinas. **c**, The area occupied by processes originating from individual microglia was quantified from whole mount retinas.

Fixed spinals cord sections (30 μm thick) from $P2Y_{12}$ $^{+/+}$$CX_3CR1$ $^{+/GFP}$ and $P2Y_{12}$ $^{-/-}$$CX_3CR1$ $^{+/GFP}$ mice were visualized by GFP epifluorescence and number of positive cell bodies within 900 x 900 μm zones. Fixed whole-mount retinas from these animals were analyzed using a Zeiss 510 meta confocal microscope and cells quantified within 370 x 370 μm zones. **c**, Confocal images from retinal sections were obtained and projections representing entire cell volumes were analyzed by drawing a boundary connecting the distal tips of all visible projections for each cell examined.

Supplemental Fig. 2 Microglia express $P2Y_{13}$ transcripts, but not detectable receptor protein. **a**, RT-PCR products were produced with primer pairs designed to specifically amplify $P2Y_{12}$ or $P2Y_{13}$ coding regions (or CX_3CR1 as a positive control) using total RNA from microglia cultured from wild-type (+/+) or $P2Y_{12}$-deficient mice. Amplification of RNA-derived reverse transcripts was confirmed by lack of signal in the absence of reverse transcriptase (RT). We also performed quantitative RT-PCR to ascertain whether $P2Y_{13}$ mRNA expression is markedly altered in $P2Y_{12}$-deficient mice, reflecting either a compensatory increase or a decrease due to the close proximity (~5 kilobases) of the $P2Y_{13}$ gene to the targeted locus. We found a slight reduction (~2-fold) in $P2Y_{13}$ signal amplified from brain tissue of $P2Y_{12}$–deficient mice compared to wild-type littermates. **b**, *In situ* hybridization of brain sections from $P2Y_{12}$ $^{+/+}$$CX_3CR1$ $^{+/GFP}$ mice using a $P2Y_{13}$-specific anti-sense probe revealed weak, but detectable signals in GFP-positive microglia. **c**, Transiently

transfected HEK923T cells expressing mouse $P2Y_{13}$ cDNA show immunoreactivity with anti-$P2Y_{13}$ antibody, whereas vector (pcDNA3)-transfected controls do not. **d**, Brain sections from $P2Y_{12}$ $^{+/+}$$CX_3CR1$ $^{+/GFP}$ mice lacked detectable immunoreactivity when stained with anti-$P2Y_{13}$ antibody. GFP fluorescence shows location of microglia. Scale bars = 50 μm.

All procedures were performed as described in Materials and Methods. The following primers pairs were used for PCR analysis:

$P2Y_{12}$: 5'-CCTCAGCCAATACCACCTTCTCCCC-3' and

5'-CGCTTGGTTCGCCACCTTCTTGTCCCTT-3'

$P2Y_{13}$ 5'-GGGACACTCGGATGACACAGCTGC-3' and

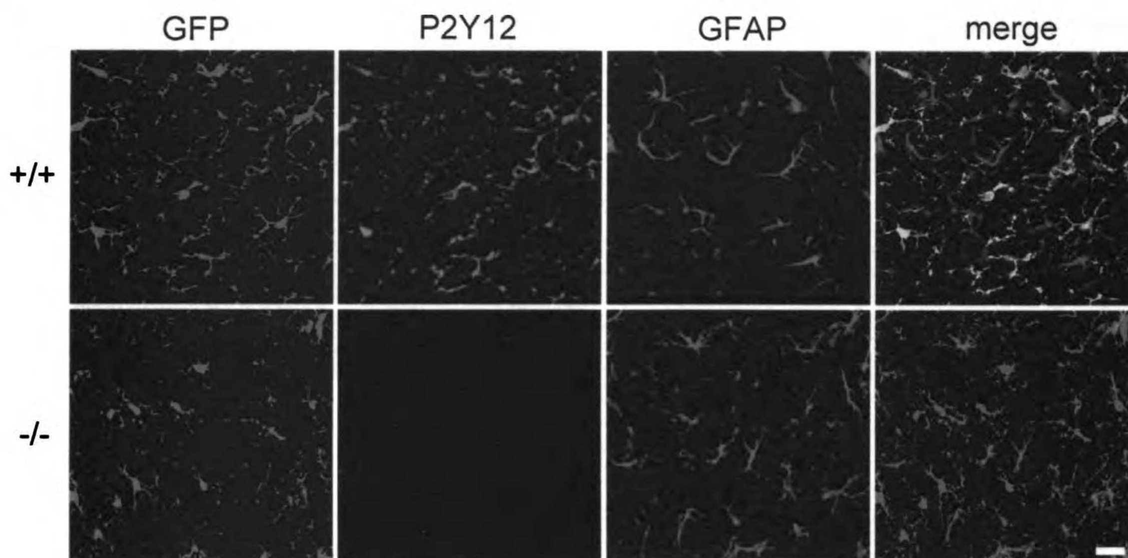
5'-GCCAGAAAGAGAGTTGCTTCTTTAGCAATAAACAGC-3'

CX_3CR1 5'- TTCACGTTCGGTCTGGTGGG-3' and

5'-GGTTCCTAGTGGAGCTAGGG-3'

Anti-$P2Y_{13}$ receptor polyclonal antibody was generated by immunizing rabbit with a synthetic peptide corresponding to the mouse $P2Y_{13}$ C-terminus (NH_2-Cys-Thr-Ala-Gly-Ser-Ser-Glu-Asp-His-His-Ser-Ser-Gln-Thr-Asp-Asn-Ile-Thr-Leu-Ala-OH; Anaspec, Inc). Antibody was affinity purified using a Sulfolink coupling gel (Pierce) to immobilize the antigenic peptide.

Figure 1



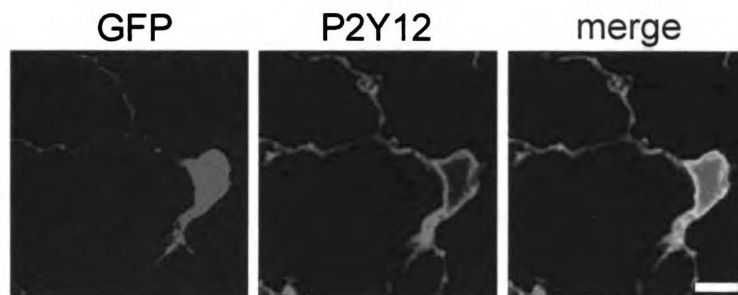


Figure 2

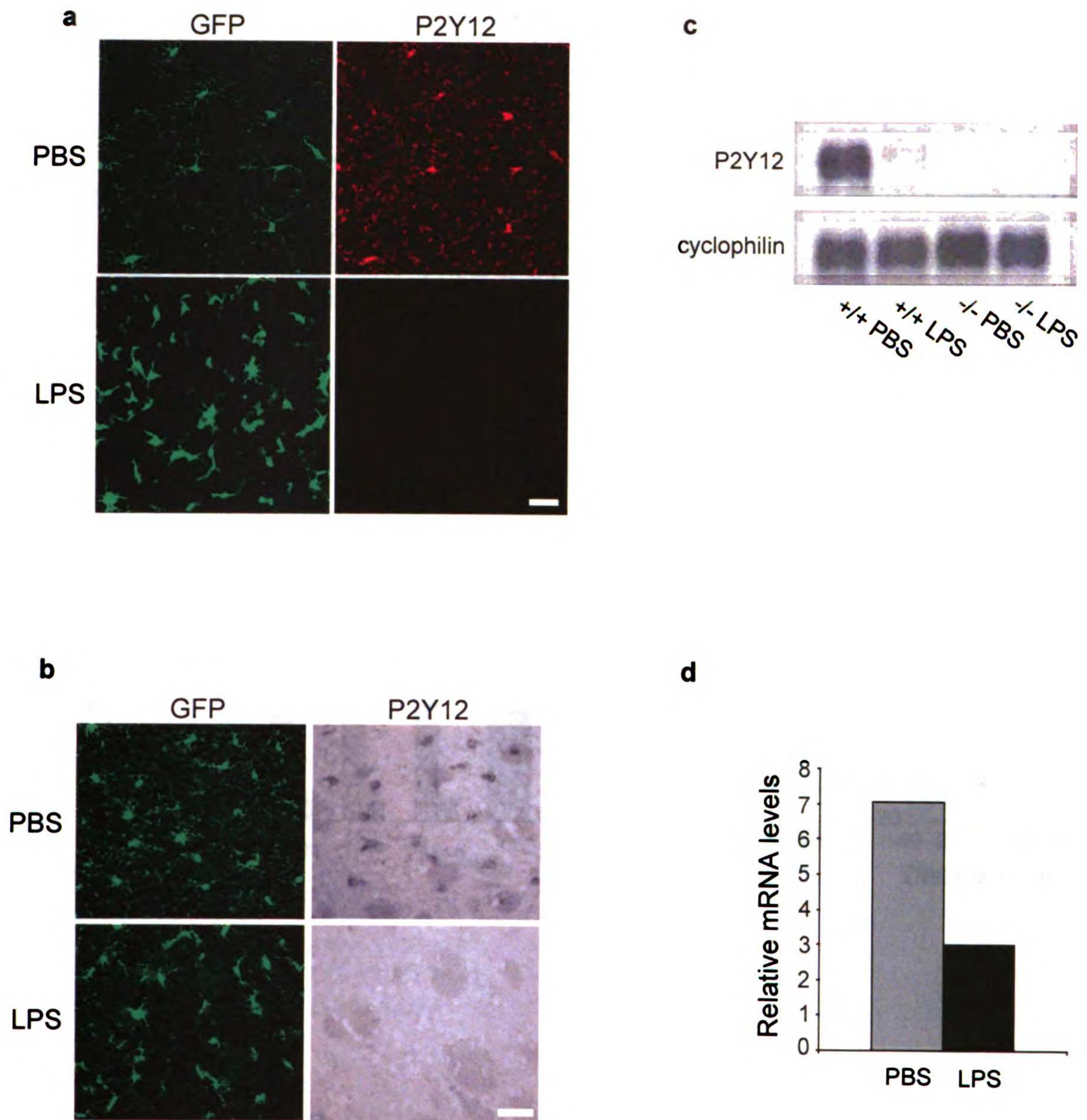


Figure 3

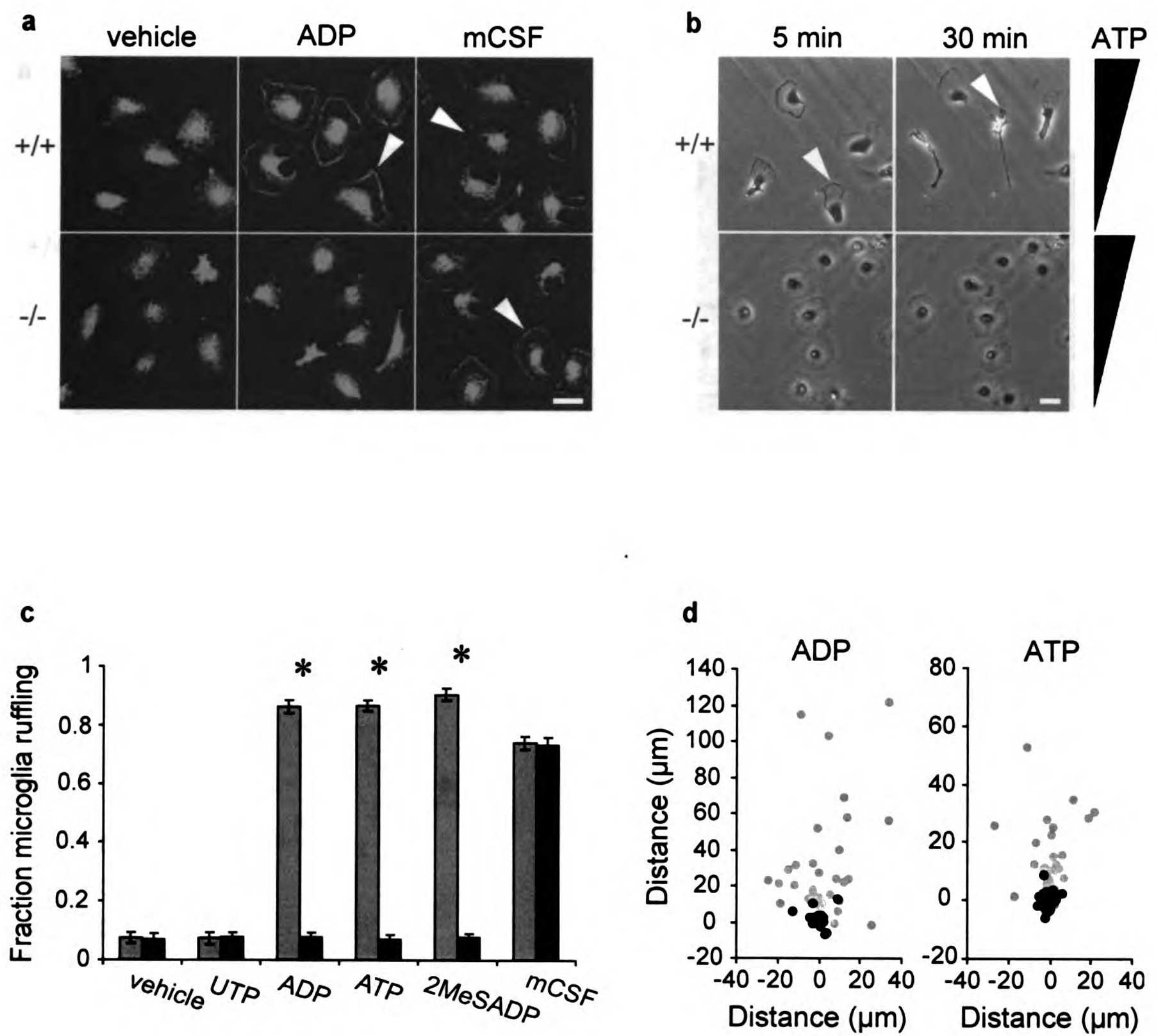


Figure 4

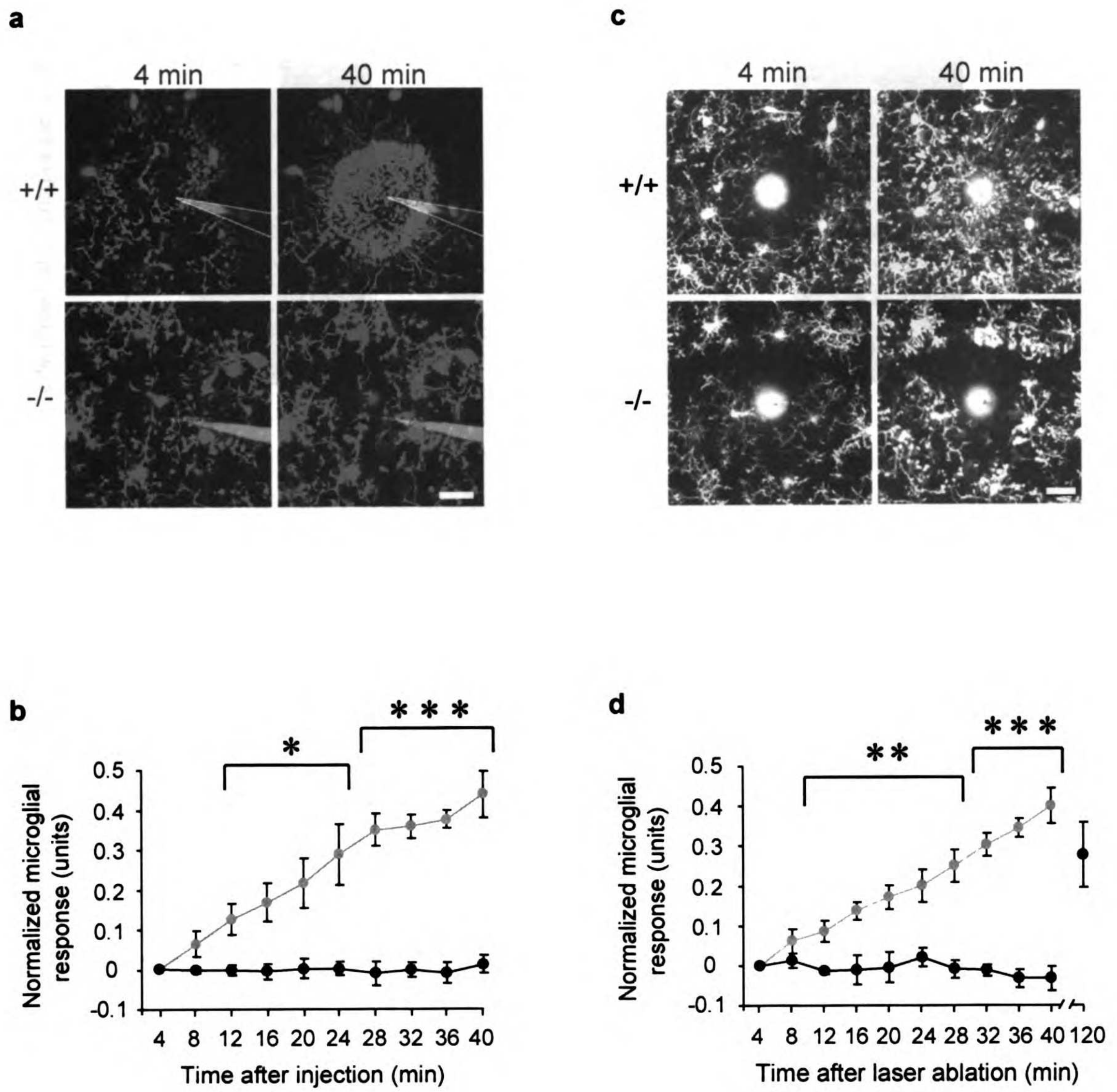


Figure S1

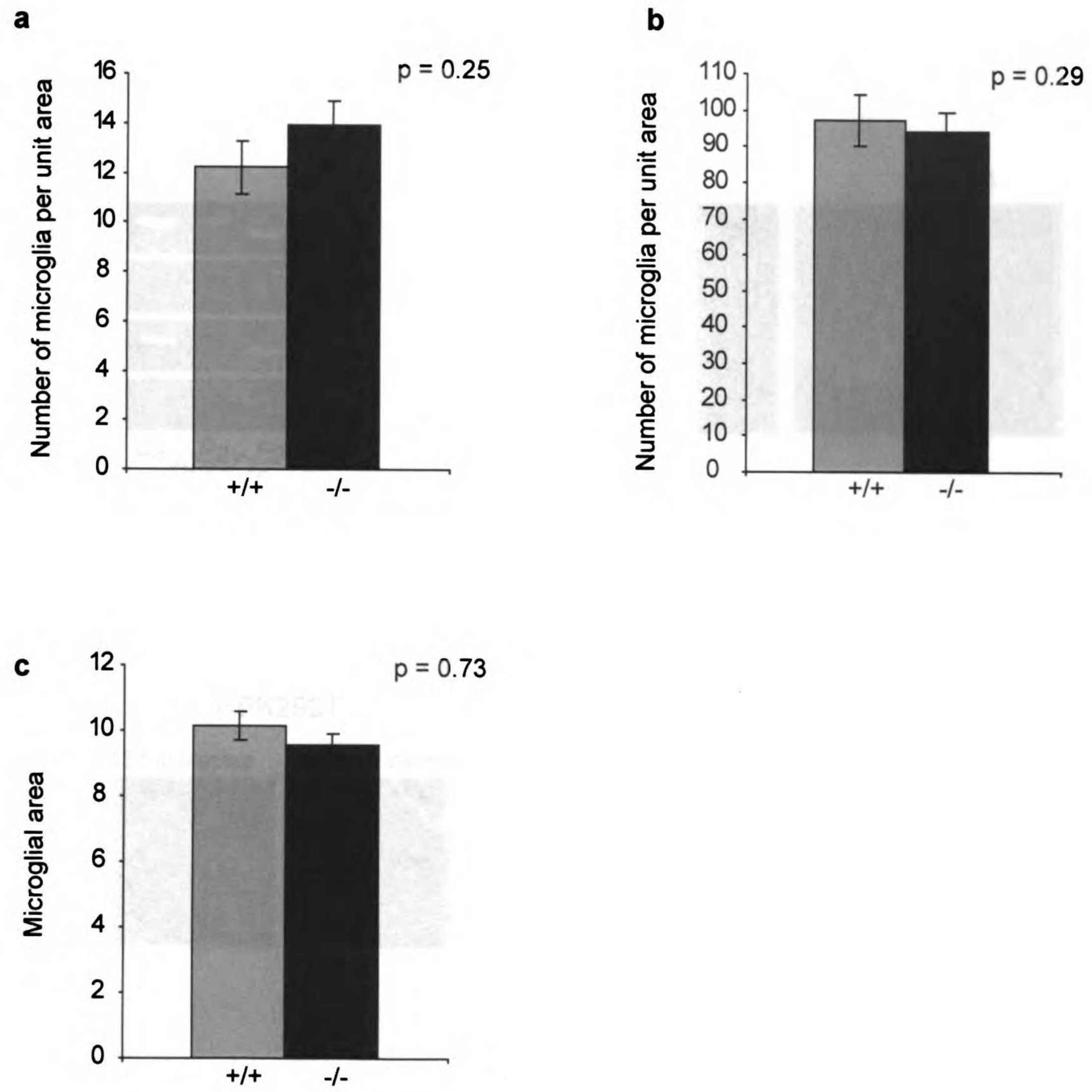
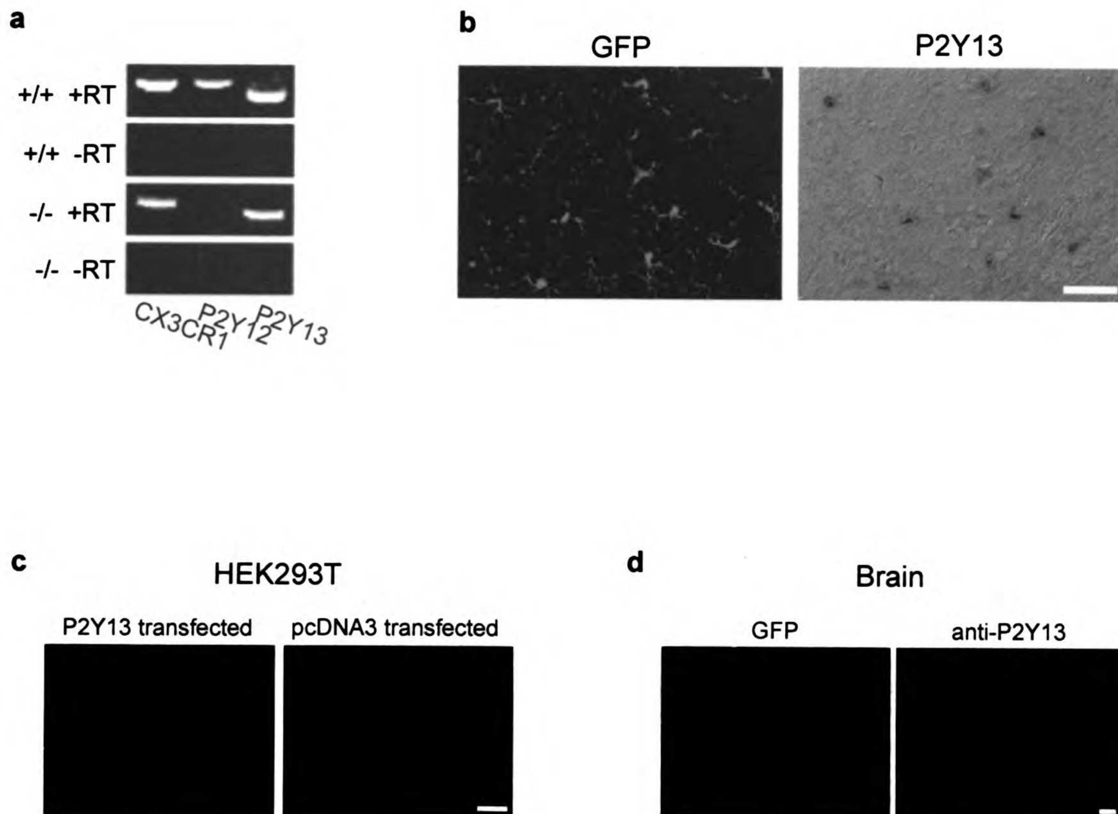


Figure S2



CHAPTER 4

Future Directions

Future Directions

The next major goal of investigating the function of $P2Y_{12}$ in microglia is to find a nervous tissue injury in which $P2Y_{12}$ deficiency alters the progression of pathology. To this end, we have examined the response of $P2Y_{12}$ knockout mice in a variety of injury models whose outcomes are hypothesized to be regulated by microglia. We have also tried to narrow our focus by avoiding models that involve massive trauma as the prolonged bleeding times noted in the $P2Y_{12}$ deficient mice may confound the analysis. Additionally, we have favored models that produce a quantifiable behavioral output so that we have a method for measuring the injury outcome at the organismal level.

The spared nerve injury model adapted for mice by the laboratory of Allan Basbaum is ideal. In this model, two branches of the sciatic nerve are severed just distal to the point at which the nerve bundle splits into three branches below the knee. The surgery is relatively bloodless and the operation site is distant from the region of CNS where microglia are seen to congregate within a day following the injury. This injury reproducibly and consistently produces a robust and prolonged hypersensitivity to mechanical force applied to the plantar surface of the injured limb[1]. Several mechanisms have been proposed to explain how this injury model, along with related human peripheral neuropathies such as sciatica, results in the development of allodynia, but a recent study implicated P2 receptors on microglia of the spinal cord as essential elements[2]. Thus, we have performed the spared nerve injury model on a cohort of our $P2Y_{12}$ deficient mice

in collaboration with Allan Basbaum's lab and compared the behavioral response to the injury with that of wildtype littermates treated in the same manner. Both groups of animals became twice as sensitive to mechanical stimulation of the injured paw within 24 hours following surgery and remained equally hypersensitive until the end of the experiment 2 weeks later. At that time, histological sections of lumbar spinal cord segments 4, 5, and 6 were prepped from the mice to enable visualization of the spinal cord region innervated by the sciatic nerve. Visual quantification of GFP-positive microglia in the dorsal horns revealed a similar increase (2-fold) in the side ipsilateral to the injury when compared to the contralateral side for both $P2Y_{12}$ knockout and wildtype mice. Thus, we do not feel there is a major contribution of $P2Y_{12}$ to the pathology produced by the spared nerve injury model.

Multiple sclerosis is a progressive and reoccurring inflammatory disease of the CNS in which the immune system attacks the myelin coat of neurons. The development and maintenance of the disease is poorly understood and our ability to treat the disorder remains minimal. Microglia have been proposed to be involved in many steps of the disease process, as have nucleotide receptors[3,4]. Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis induced by immunizing mice with a fragment of myelin oligodendrocyte glycoprotein. This produces a long lasting disease state in the animals characterized by relapsing bouts of variable paralysis that can be quantified using a standardized scoring method[5]. We have performed this model

on a large group of our $P2Y_{12}$ knockout mice and their wildtype littermates with Larry Steinman's lab at Stanford University. Approximately ten days following the initial immunization, both groups of mice started to show outward signs of the disease progression and the elevated disease scores were maintained equally in the two groups for the remainder of the testing period (40 days) at which time they were sacrificed for histological examination of the CNS tissue. Both $P2Y_{12}$ and wildtype mice exhibited an equivalent distribution of lesion sites scattered throughout the white matter that were characterized by the presence of GFP-positive cells bearing the morphology of activated microglia. These cells were also positive for CD11b and we assume they represent microglia attracted to the plaques. However, we noted that these were not immunoreactive for $P2Y_{12}$. This suggests that $P2Y_{12}$ is down regulated by microglia following activation. Nevertheless, we do not believe that $P2Y_{12}$ plays a major role in the development of EAE.

We have also tested the $P2Y_{12}$ deficient animals in other models of CNS injury. These include the classic stab-wound model, intraocular herpes virus infection, and a brain tumor model in which mouse glioma cells are implanted into the striatum. In all cases we have not found a robust difference between $P2Y_{12}$ knockout mice and their wildtype littermates in progression of the pathology. One problem with our analysis of injury models is that it involves only crude histological examination of injury sites, usually at the termination of the experiment. We may need to examine additional parameters of cell morphology,

histological markers, or CNS injury at several time points before we will detect a phenotype due to $P2Y_{12}$ deficiency. The collaboration with Wenbiao Gan's lab has taught us that we should possibly focus our attention on time points immediately following CNS injury. It is clear that microglia of the $P2Y_{12}$ deficient animals are delayed in their ability to rapidly extend processes toward sites of laser injury, so these mice provide an opportunity to determine what, if anything, the microglia are doing once they arrive at injury sites.

Next, we are planning to examine the $P2Y_{12}$ deficient mice in a stroke model because there is literature that indicates nucleotides and microglia may be important mediators of the neuronal loss following an ischemic insult[6]. If there are differences due to lack of $P2Y_{12}$ it may be difficult to separate the contribution of platelets from the contribution of microglia, as this type of injury involves the cardiovascular system within the nervous system. There are three main ways one could deal with these issues. One method would be to give the animals clopidogrel prior to the injury which should only inactivate $P2Y_{12}$ on circulating platelets and not on resident microglia of the CNS. This would serve to make the wildtype animals equivalent to the knockout animals with respect to platelet function. The second way to separate effects of $P2Y_{12}$ deficiency in platelets with those of $P2Y_{12}$ deficiency in microglia would be to replace the expression of $P2Y_{12}$ in the knockout mice using a platelet specific promoter. There are a few selective, robust, and compact promoters to choose from that should be straightforward to use in the engineering of a classic transgenic mouse.

Another method to rescue $P2Y_{12}$ expression in the platelets would be to transplant bone marrow stem cells from $P2Y_{12}$ wildtype mice into $P2Y_{12}$ knockout mice. This presumes that the turnover of microglia within the CNS is much slower than the turnover of platelets in the periphery and would allow one to consider the transplant recipients as $P2Y_{12}$ wildtypes with respect to platelet function. In healthy rodents receiving bone marrow transplants from labeled donors, the appearance of tagged microglia in the CNS is a slow process [7,8]. However, after under conditions of CNS duress, the microglia that populate injury sites tend to be newly derived from the donor bone marrow stem cells[9,10]. Thus, it would probably be necessary to focus on time points immediately following CNS injury to limit the infiltration of $P2Y_{12}$ expressing microglia into the damaged nervous tissue of $P2Y_{12}$ knockout mice after they receive wildtype bone marrow transplants. Collecting transplant marrow from GFP labeled mice would allow one to visualize the cells if they enter the CNS.

In the injury models we have looked at thus far, we find that $P2Y_{12}$ expression is reduced or absent in presumptive microglia at sites of CNS injury. In addition, we do not believe other immune cells outside of the CNS express $P2Y_{12}$; although it is possible we have overlooked a rare niche population. Thus, within the immune system it seems that $P2Y_{12}$ expression is only turned on in microglia of the healthy CNS. It is intriguing to imagine how this might be accomplished. One possibility is that there is a factor secreted by the intact brain that causes

microglia to produce $P2Y_{12}$ transcripts. The other possibility is that once nascent microglia enter the CNS, they are shielded from an inhibitory substance that normally represses $P2Y_{12}$ transcription. The CNS is one of the few locations of the body that is considered "immunoprivileged" and it may be that the expression of $P2Y_{12}$ by microglia is due to this unique immunological state shared by the CNS, the anterior chamber of the eye, and the placenta[11]. It would be interesting to examine placental tissue for immune cells expressing $P2Y_{12}$.

Additionally, we have had difficulty detecting $P2Y_{12}$ protein in cultured microglia, although we can detect $P2Y_{12}$ transcripts by RT-PCR and the morphological response to ADP application by these cells suggest the presence of functional $P2Y_{12}$ receptors. The cultured microglia may be a reasonable system in which to investigate the regulation of $P2Y_{12}$ expression in microglia. One of the first experiments might be to inject microglia cultured from a GFP positive mouse into the CNS of an unlabelled animal to see if the GFP positive microglia become immunopositive for $P2Y_{12}$ once exposed to the permissive environment of the intact CNS. Anther useful tool for dissecting the transcriptional regulation of $P2Y_{12}$ would be to target a genetically encoded marker such as GFP to the $P2Y_{12}$ locus so that the expression would be under control of the $P2Y_{12}$ transcriptional elements. If it becomes clear that the CNS produces a substance that activates $P2Y_{12}$ expression or that the periphery produces a factor inhibitory to $P2Y_{12}$ transcription, it may be possible to biochemically purify and identify the factor using GFP fluorescence as a readout. It is also tantalizing to wonder if the

reduced expression of $P2Y_{13}$ in microglia of $P2Y_{12}$ knockout mice is due to alterations of chromosomal structure at targeted allele limiting access of the $P2Y_{13}$ promoter to a common regulatory element driving expression of both transcripts in microglia.

One of the hallmarks of $P2Y_{12}$ is its sensitivity to inactivation by compounds that modify cysteine residues. This is hypothesized to be the mechanism by which clopidogrel inactivates $P2Y_{12}$ receptors on platelets, thereby inhibiting thrombotic events[12]. Of course, it is likely that clopidogrel also has structural requirements that further target $P2Y_{12}$ specifically, but the evidence that the mercury compound, PCMBS, will ablate $P2Y_{12}$-mediated signals in oocytes without affecting other GPCR-mediated signals suggests that $P2Y_{12}$ receptor activity is dependent upon free mercaptans within the protein. There are four extracellular cysteine residues on P2Y1 that are required for function[13]. They are also present in $P2Y_{12}$ and a recent study has proposed that the cysteines on the first and second extracellular loops are required for $P2Y_{12}$ expression, while the cysteines on the amino terminus and third extracellular loop seem to be required for PCMBS to inhibit the receptor[14]. P2Y1 is a reasonable choice for comparison with $P2Y_{12}$ because they share a similar pharmacological profile and are somewhat related at the sequence level. Additionally, they are both expressed in platelets and it seems that clopidogrel selectively inactivates $P2Y_{12}$ proteins in this setting. However, the identification of $P2Y_{13}$ may provide an even better candidate for structure/function studies in unison with $P2Y_{12}$. The $P2Y_{13}$ protein

is strikingly similar to $P2Y_{12}$ in the parameters of sequence conservation, receptor coupling, and pharmacology. In our initial experiments involving $P2Y_{13}$ heterologously expressed in *Xenopus* oocytes, we find that $P2Y_{13}$ is resistant to inactivation by PCMBS. This indicates that $P2Y_{13}$ may represent an ideal protein sequence to compare with $P2Y_{12}$ in the hopes of uncovering the mechanism of clopidogrel's action.

Nitric oxide is widely recognized as having antiplatelet function although the process by which this gaseous messenger inhibits clot formation is not completely understood[15]. A general mechanism for nitric oxide's actions on proteins is proposed to be *S*-nitrosylation, the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteines[16]. Thus, it is possible that nitric oxide inhibits the clotting process by inactivating $P2Y_{12}$ via cysteine modification, in a manner reminiscent of clopidogrel. We currently have methods to liberate nitric oxide in our oocyte perfusion system and it will be interesting to test if $P2Y_{12}$ signaling exhibits inhibition in the presence of nitric oxide. Additionally, macrophagic cells of the immune system produce nitric oxide in response to activation as a means of destroying pathogens with cytotoxic damage. LPS is an effective liberator of nitric oxide from microglia[17] and has been reported to abolish ADP-mediated calcium signals in cultured microglia[18]. Although it is not clear how ADP activates calcium release in these cells, it is important to note that the pharmacology of the response lost after LPS treatment shares similarity with that of $P2Y_{12}$. When combined with the knowledge that

LPS treatment causes loss of $P2Y_{12}$ expression in microglia, there is reason to believe that activation of microglia may limit $P2Y_{12}$ function via S-nitrosylation of labile cysteines following nitric oxide release. The next experiment to be done on this front would be to treat cultured microglia with LPS and/or nitric oxide to examine if the chemotactic response to nucleotides is lost.

$P2Y_{12}$ represents the first functionally characterized member of a novel family of G-protein coupled receptors[19]. In the human genome, this family of five genes is arranged sequentially on chromosome three with $P2Y_{12}$ in the lead (www.ensembl.org). The $P2Y_{13}$ gene is located only a few kilobases downstream of $P2Y_{12}$. The next gene on the chromosome, GPR87, is closely related to $P2Y_{12}$ at the sequence level and hypothesized to be another Gi-coupled nucleotide receptor[20]. The next transcriptional unit corresponds to $P2Y_{14}$, which was identified as a Gi-coupled receptor for UDP-glucose[21]. Interestingly, this receptor is found on bone marrow hematopoietic cells and thought to mediate their chemotaxis towards conditioned media from bone marrow stroma[22]. Finally, the gene for GPR171 closely follows that of $P2Y_{14}$. This hypothetical G protein-coupled receptor shares sequence homology with the four other receptors whose genes are located upstream on the chromosome[23]. It is highly likely that this intimate juxtaposition of receptor genes whose predicted coding sequences are so closely related indicates that they are the products of relatively recent gene duplication events. Not only do they share sequence homology and chromosomal proximity, but it is also beginning to look like they

also share similar functions and expression patterns. With respect to the functionally characterized members $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, they all signal through pertussis toxin sensitive G proteins[19]. This is in contrast to other members of the P2Y class of receptors that primarily signal through Gq to liberate calcium from internal stores. In general, it seems that this new family of receptors tends to be expressed by bone marrow-derived cells[24-26]. Thus, one could hypothesize that this new class of P2Y receptors has evolved to allow detection of tissue injury by cells of the cardiovascular and immune systems.

References

1. Shields, S. D., Eckert, W. A., 3rd & Basbaum, A. I. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *J Pain* **4**, 465-70 (2003).
2. Tsuda, M. et al. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* **424**, 778-83 (2003).
3. Tsutsui, S. et al. A1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis. *J Neurosci* **24**, 1521-9 (2004).
4. Heppner, F. L. et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat Med* **11**, 146-52 (2005).
5. Steinman, L. Assessment of animal models for MS and demyelinating disease in the design of rational therapy. *Neuron* **24**, 511-4 (1999).
6. Volonte, C. et al. Extracellular ATP and neurodegeneration. *Curr Drug Targets CNS Neurol Disord* **2**, 403-12 (2003).
7. Albini, T. A. et al. Microglial stability and repopulation in the retina. *Br J Ophthalmol* **89**, 901-3 (2005).
8. Vallieres, L. & Sawchenko, P. E. Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. *J Neurosci* **23**, 5197-207 (2003).
9. Yagi, T. et al. Fate of donor hematopoietic cells in demyelinating mutant mouse, twitcher, following transplantation of GFP+ bone marrow cells. *Neurobiol Dis* **16**, 98-109 (2004).

10. Villeneuve, J., Tremblay, P. & Vallieres, L. Tumor necrosis factor reduces brain tumor growth by enhancing macrophage recruitment and microcyst formation. *Cancer Res* **65**, 3928-36 (2005).

11. Barker, C. F. & Billingham, R. E. Immunologically privileged sites. *Adv Immunol* **25**, 1-54 (1977).

12. Dorsam, R. T., Murugappan, S., Ding, Z. & Kunapuli, S. P. Clopidogrel: Interactions with the P2Y12 Receptor and Clinical Relevance. *Hematology* **8**, 359-65 (2003).

13. Hoffmann, C., Moro, S., Nicholas, R. A., Harden, T. K. & Jacobson, K. A. The role of amino acids in extracellular loops of the human P2Y1 receptor in surface expression and activation processes. *J Biol Chem* **274**, 14639-47 (1999).

14. Ding, Z., Kim, S., Dorsam, R. T., Jin, J. & Kunapuli, S. P. Inactivation of the human P2Y12 receptor by thiol reagents requires interaction with both extracellular cysteine residues, Cys17 and Cys270. *Blood* **101**, 3908-14 (2003).

15. Gustavo, R., Stefania, M. & Paolo, G. Nitric oxide and its antithrombotic action in the cardiovascular system. *Curr Drug Targets Cardiovasc Haematol Disord* **5**, 65-74 (2005).

16. Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E. & Stamler, J. S. Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol* **6**, 150-66 (2005).

17. Wang, A. L. et al. Minocycline inhibits LPS-induced retinal microglia activation. *Neurochem Int* **47**, 152-8 (2005).

18. Moller, T., Kann, O., Verkhratsky, A. & Kettenmann, H. Activation of mouse microglial cells affects P2 receptor signaling. *Brain Res* **853**, 49-59 (2000).

19. Abbracchio, M. P. et al. Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* **24**, 52-5 (2003).

20. Lee, D. K. et al. Discovery and mapping of ten novel G protein-coupled receptor genes. *Gene* **275**, 83-91 (2001).

21. Chambers, J. K. et al. A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275**, 10767-71 (2000).

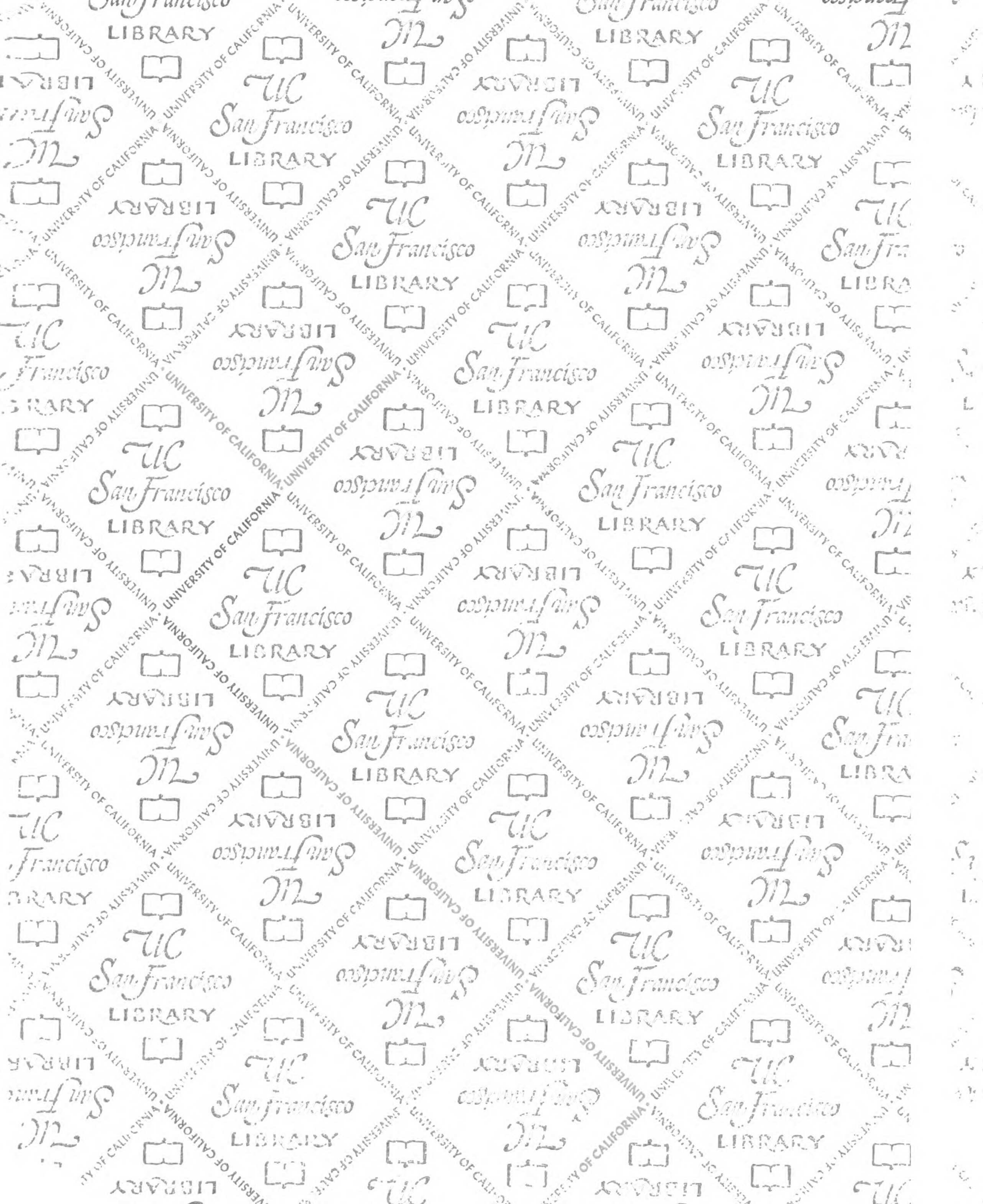
22. Lee, B. C. et al. P2Y-like receptor, GPR105 (P2Y14), identifies and mediates chemotaxis of bone-marrow hematopoietic stem cells. *Genes Dev* **17**, 1592-604 (2003).

23. Wittenberger, T., Schaller, H. C. & Hellebrand, S. An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. *J Mol Biol* **307**, 799-813 (2001).

24. Zhang, F. L. et al. P2Y(13): identification and characterization of a novel Galphai-coupled ADP receptor from human and mouse. *J Pharmacol Exp Ther* **301**, 705-13 (2002).

25. Freeman, K. et al. Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. *Genomics* **78**, 124-8 (2001).

26. Charlton, M. E., Williams, A. S., Fogliano, M., Sweetnam, P. M. & Duman, R. S. The isolation and characterization of a novel G protein-coupled receptor regulated by immunologic challenge. *Brain Res* **764**, 141-8 (1997).



For reference

Not to be taken from the room.