Title
The P2Y12 receptor regulates microglial activation by extracellular nucleotides

Author
Haynes, Sharon E

Publication Date
2006

Peer reviewed|Thesis/dissertation

The P2Y12 receptor regulates microglial activation by extracellular nucleotides

by

Sharon E. Haynes

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

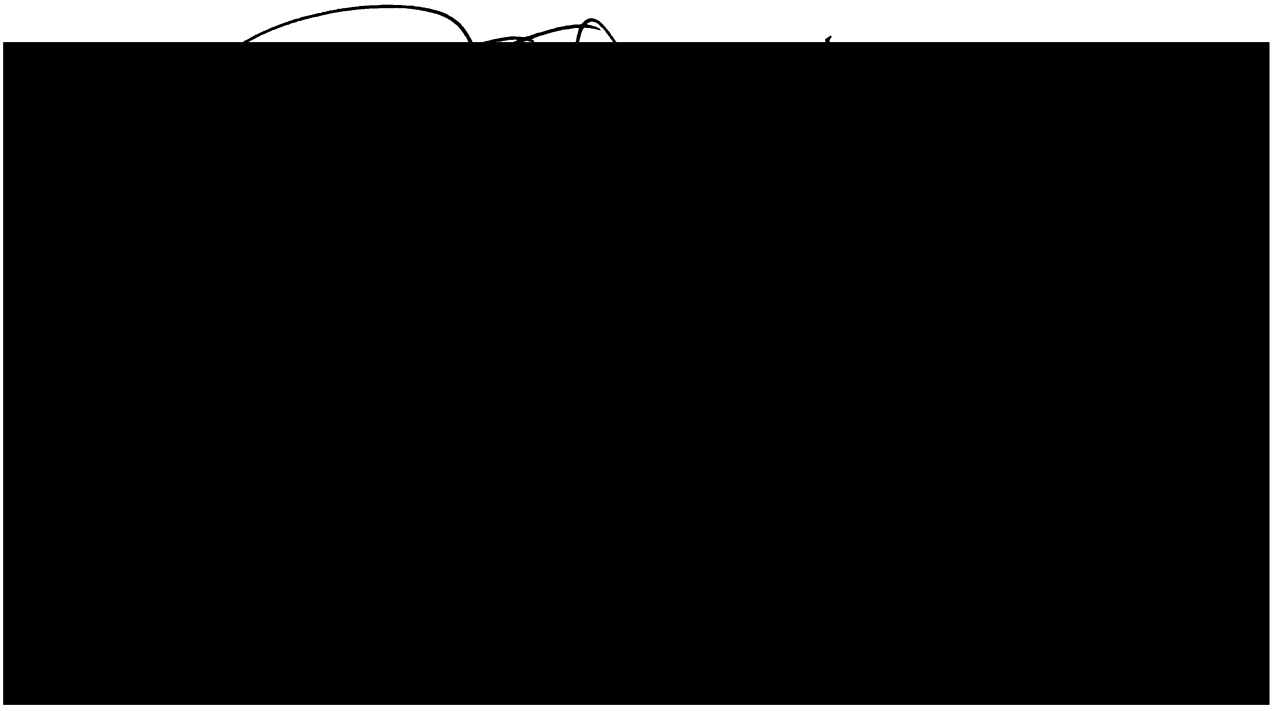
Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



Acknowledgments

This study would not have been possible without the help of many talented scientists, for whom I am extremely grateful. First, I would like to thank my research advisor David Julius for providing a stimulating lab environment, and for always offering his assistance and guidance. I would also like to thank my thesis committee, Rick Brown, Li Gan, and Mark von Zastrow, who have been extremely supportive and generous of their time. I am forever indebted to Gunther Hollopeter who taught me many things, from mouse genetics to Middle Eastern cuisine. Gunther brought a lot of life into the Julius Lab and for that he will always be remembered. It has been a great honor to work with my collaborators, Wenbiao Gan at New York University and Michael E. Dailey at the University of Iowa. I greatly appreciate their enthusiasm for this project, and I am so thankful for all that they have taught me. Martha Chase and Amanda Weyerbacher have been really great friends, and I am very lucky to have them both in my life. They are the reason that I have made it through grad school without losing my sanity. Lastly, I am forever grateful to Phil Meakin, my sister Lancia Herzog, and my parents Bill and Lettie Haynes for their unconditional love and support. Their continued belief in my abilities has given me the strength and courage to pursue my dreams.

"The $P2Y_{12}$ receptor regulates microglial activation by extracellular nucleotides" has been reprinted by permission from Macmillan Publishers Ltd: Nature Neuroscience 9, 1512-9, (2006).

Permission

NATURE PUBLISHING GROUP LICENSE

TERMS AND CONDITIONS

Dec 12, 2006

This is a License Agreement between Sharon E Haynes ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

License Number	1606750346548
License date	Dec 12, 2006
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Neuroscience
Licensed content title	The P2Y12 receptor regulates microglial activation by extracellular nucleotides
Licensed content author	Sharon E Haynes, Gunther Hollopeter, Guang Yang, Dana Kurpius, Michael E Dailey, Wen-Biao

Gan, ...

Volume number	9
Issue number	12
Pages	1512-1519
Year of publication	2006
Portion used	Full paper
Requestor type	Student
Type of Use	Thesis / Dissertation
Total	$0.00

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, **and for no other use**, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was

reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

   Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).

   NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the

figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   For AOP papers, the credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of up to a whole article do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Abstract

The $P2Y_{12}$ receptor regulates microglial activation by extracellular nucleotides

Sharon Haynes

Microglia are primary immune sentinels of the central nervous system (CNS). Following injury, these cells migrate or extend processes towards sites of tissue damage. CNS injury is accompanied by the release of nucleotides, serving as signals for microglial activation or chemotaxis. Microglia express several purinoceptors, including a G_i-coupled subtype that has been implicated in ATP/ADP-mediated migration *in vitro*. Here we show that microglia from mice lacking G_i-coupled $P2Y_{12}$ receptors exhibit normal baseline motility but are unable to polarize, migrate, or extend processes towards nucleotides *in vitro* or *in vivo*. Microglia in *P2ry12*$^{-/-}$ mice display significantly diminished directional branch extension towards sites of cortical damage in the living mouse. 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 microglial chemotaxis at early stages of the response to local CNS injury.

Reprinted by permission from Macmillan Publishers Ltd:

Nature Neuroscience 9, 1512-9, (2006)

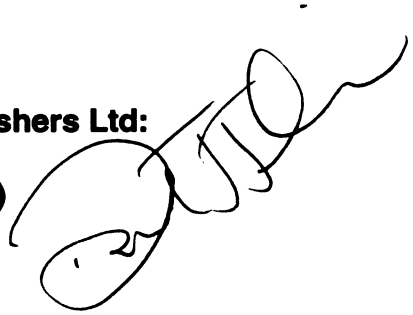


Table of Contents

List of Figures

CHAPTER 2

CHAPTER 3

CHAPTER 1

INTRODUCTION

INTRODUCTION

Microglia are the primary mediators of the immune response that occurs following central nervous system (CNS) injury or disease. They are derived from blood monocytes, sharing a developmental lineage with peripheral macrophages, and invade the central nervous system during embryonic development[1]. In the healthy adult brain, microglia comprise 10% of total brain cells[2] and are found in a resting, highly ramified state in which they keep a close surveillance over their local environment. In this "un-activated" state, microglia are rapidly projecting and retracting their elaborate ramifications, possibly as a means of surveying their local environment[3,4]. When injury or disease strikes, microglia become "activated" and migrate or send cellular projections towards the site of damage[3-5]. In this activated state, they take on a macrophage-like phenotype[5] and are able to phagocytose debris, release cytokines or inflammatory factors, and present antigens to infiltrating T cells[6,7]. Microglia are thought to be both protective and deleterious to the CNS because the neuromodulating factors they secrete can promote either cell survival or cell death[8]. It has also been suggested that microglia facilitate synapse formation and reconstruction[9] and modulate pain sensitivity following nerve damage[10].

Microglia use numerous signaling mechanisms to monitor the physical condition of their local environment[2]. For example, extracellular nucleotides released at an

injury site may activate purinoceptors on microglia. Some aspects of microglial response to injury can be mimicked by injection of ATP, or attenuated by broad-spectrum inhibitors of purinergic signalling[3], suggesting that nucleotides function as regulators of microglial behavior *in vivo*. Specifically, it has been shown that cortical tissue damage inflicted by a two-photon laser induces local microglia to extend cellular projections towards the injury. This response can be abrogated by a saturating concentration of extracellular ATP[3], implicating that a nucleotide gradient is important to elicit this behavior.

Purinergic receptors, designated P, are subdivided into classes depending on their mode of action. P1 receptors are activated by adenine and uridine, whereas P2 receptors are activated by ATP, UTP and their derivatives. The postscript X designates those that are ionotrophic, or ligand-gated ion-channels, and Y designates those that are metabotrophic, or G-protein coupled receptors[11,12]. Nucleotides activate microglia via a number of purinoceptors on the cell surface such as $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_{12}$[13]. Although the roles of these purinoceptors are poorly understood, it has been shown that $P2X_4$ may play a role in tactile allodynia after nerve injury through ATP-evoked BDNF release[14,15] and $P2X_7$ may induce the release of TNF-α and IL-1β[16]. Low levels of ATP or ADP exposure induce microglia to release neuroprotective factors, such as plasminogen, whereas high levels induce neurotoxic factors, such as nitric oxide, although the receptor(s) mediating this response is unknown[13].

Extracellular concentrations can rise rapidly and dramatically when injured cells lose membrane integrity and release their cytosolic contents[17]. ATP is released from neurons, astroglia, and microglia[18], and functions in cell-cell communication. Regulated nucleotide release from neurons and astrocytes may occur by exocytosis or through various ATP pumps such as the multi-drug resistance (MDR) protein or cystic fibrosis transmembrane conductance regulator (CFTR)[19,20]. ATP may be released from microglia following activation and may serve as a positive feedback signal[21]. The role of nucleotides as autocrine and paracrine cell modulators has been well documented in the immune, nervous, and cardiovascular systems. In the immune system, extracellular ATP activates mast cell degranulation as well as induces the formation of a nonselective membrane pore permeable to molecules up to 900Da in lymphocytes and macrophages[20]. There is evidence that suggests ATP may also be utilized as a chemotactic agent in dendritic cells, hematopoietic cells that share a developmental lineage with microglia and macrophages[22]. In the nervous system, purinoceptors on neurons mediate ATP-dependent membrane depolarization and action potential firing[23].

It has recently been shown that ATP or ADP can evoke membrane ruffling (actin polymerization) and directed migration of cultured microglia through a pertussis toxin-sensitive signalling pathway, thus implicating G_i-coupled receptor(s)-mediated inhibition of cyclic AMP synthesis[24]. The vast majority of metabotropic P2Y receptors transduce their signals through G_q/phospholipase C pathways[25].

However, in 2001 the first G_i-coupled purinergic receptor was identified, termed $P2Y_{12}$, and was shown to be expressed in glial cells of the brain[26,27]. As such, $P2Y_{12}$ is an attractive candidate for mediating morphological responses of microglia to extracellular nucleotides.

Although ADP is the primary agonist of $P2Y_{12}$, ATP is a partial agonist; thus ATP acts as an antagonist when receptor reserves are low (perhaps in platelets) and a weak agonist when receptor reserves are high[28]. Activation of $P2Y_{12}$ induces the dissociation of heterotrimeric G-protein subunits so that G_α may inhibit adenylyl cyclase and $G_{\beta\gamma}$ may activate inwardly rectifying K^+ (K_{IR}) channels[26]. In fact, there has been evidence that microglia express K_{IR} channels[29], although the role of this channel in microglial physiology is not understood. In addition, $P2Y_{12}$ has been shown to activate Rac GTPase, which in turn activates actin polymerization at the leading edge of a migrating cell[24]. Recent evidence suggests that $P2Y_{12}$ desensitizes after activation via a kinase-dependent mechanism in platelets[30] and is localized to lipid rafts in the membrane[31].

The $P2Y_{12}$ receptor was initially identified on platelets, where it regulates their conversion from the inactive to active state during thrombosis[26,32,33]. Thus, growth of a nascent clot is dependent on the release of ADP (and other factors such as thromboxane A_2)[34] from activated platelets or damaged red blood cells, thereby promoting a feed-forward mechanism to recruit platelets into the active, clotting competent state. Indeed, peripherally active $P2Y_{12}$ antagonists, such as

ticlopidine (Ticlid) and clopidogrel (Plavix), block platelet activation and reduce the risk of recurrent heart attack or stroke[35]. Moreover, mutations in the $P2Y_{12}$ receptor (in humans or mice) result in prolonged bleeding times due to the reduced ability of $P2Y_{12}$-deficient platelets to change shape and undergo coagulation in response to ADP released after vascular injury[33].

To determine the physiological role of $P2Y_{12}$ in microglia, we, in collaboration with researchers formerly of COR Therapeutics, generated mice genetically engineered to lack $P2Y_{12}$ receptors. In order to facilitate imaging studies of microglia, we crossed *P2ry12*$^{-/-}$ mice with a line of mice genetically engineered to express green fluorescent protein (GFP) specifically in microglia and their precursor cells. These mice, a gift from Dr. Dan Littman at New York University, were made by replacing the gene for fractalkine receptor CX_3CR1 with the gene encoding GFP. *Cx3cr1*$^{GFP/GFP}$ mice exhibit a normal microglial response when challenged with sciatic nerve lesion[36], allowing us to use mice that are heterozygous for CX_3CR1 in some of our migration studies knowing a deficiency in microglial response will not be due to the genetic disruption of this receptor.

In light of the importance of this receptor in platelet activation, we asked whether $P2Y_{12}$ might play a similar role in microglia by contributing to their ability to respond to injury through their capacity to detect nucleotide release. 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 address this question. By examining microglial behavior in a variety of *in vitro* and *in vivo* systems, we now show that $P2Y_{12}$ is, indeed, an essential component of the signalling pathway through which extracellular purines promote directed microglial movement following CNS injury. Moreover, our findings suggest that, as in platelets, signalling through $P2Y_{12}$ is most relevant during early stages of the microglial activation process.

References

1. Kaur, C., Hao, A. J., Wu, C. H. & Ling, E. A. Origin of microglia. *Microsc Res Tech* **54**, 2-9 (2001).
2. Polazzi, E. & Contestabile, A. Reciprocal interactions between microglia and neurons: from survival to neuropathology. *Rev Neurosci* **13**, 221-42 (2002).
3. Davalos, D. et al. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* **8**, 752-8 (2005).
4. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314-8 (2005).
5. Stence, N., Waite, M. & Dailey, M. E. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* **33**, 256-66 (2001).
6. Berman, N. E. et al. Microglial activation and neurological symptoms in the SIV model of NeuroAIDS: association of MHC-II and MMP-9 expression with behavioral deficits and evoked potential changes. *Neurobiol Dis* **6**, 486-98 (1999).
7. Zhang, C. & Tso, M. O. Characterization of activated retinal microglia following optic axotomy. *J Neurosci Res* **73**, 840-5 (2003).
8. Giulian, D., Vaca, K. & Corpuz, M. Brain glia release factors with opposing actions upon neuronal survival. *J Neurosci* **13**, 29-37 (1993).

9. Bruce-Keller, A. J. Microglial-neuronal interactions in synaptic damage and recovery. *J Neurosci Res* **58**, 191-201 (1999).

10. Watkins, L. R., Milligan, E. D. & Maier, S. F. Spinal cord glia: new players in pain. *Pain* **93**, 201-5 (2001).

11. North, R. A. & Surprenant, A. Pharmacology of cloned P2X receptors. *Annu Rev Pharmacol Toxicol* **40**, 563-80 (2000).

12. von Kugelgen, I. & Wetter, A. Molecular pharmacology of P2Y-receptors. *Naunyn Schmiedebergs Arch Pharmacol* **362**, 310-23 (2000).

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

14. Coull, J. A. et al. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* **438**, 1017-21 (2005).

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

16. 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).

17. Cook, S. P. & McCleskey, E. W. Cell damage excites nociceptors through release of cytosolic ATP. *Pain* **95**, 41-7 (2002).

18. Franke, H. et al. P2 receptor-types involved in astrogliosis in vivo. *Br J Pharmacol* **134**, 1180-9 (2001).

19. Guthrie, P. B. et al. ATP released from astrocytes mediates glial calcium waves. *J Neurosci* **19**, 520-8 (1999).

20. Brake, A. J. & Julius, D. Signaling by extracellular nucleotides. *Annu Rev Cell Dev Biol* **12**, 519-41 (1996).

21. Ferrari, D. et al. ATP-mediated cytotoxicity in microglial cells. *Neuropharmacology* **36**, 1295-301 (1997).

22. Idzko, M. et al. Nucleotides induce chemotaxis and actin polymerization in immature but not mature human dendritic cells via activation of pertussis toxin-sensitive P2y receptors. *Blood* **100**, 925-32 (2002).

23. Khakh, B. S. Molecular physiology of P2X receptors and ATP signalling at synapses. *Nat Rev Neurosci* **2**, 165-74 (2001).

24. 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).

25. Burnstock, G. P2 purinoceptors: historical perspective and classification. *Ciba Found Symp* **198**, 1-28; discussion 29-34 (1996).

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

27. 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).

28. Nicholas, R. A. Identification of the P2Y(12) receptor: a novel member of the P2Y family of receptors activated by extracellular nucleotides. *Mol Pharmacol* **60**, 416-20 (2001).

29. Boucsein, C., Kettenmann, H. & Nolte, C. Electrophysiological properties of microglial cells in normal and pathologic rat brain slices. *Eur J Neurosci* **12**, 2049-58 (2000).

30. Hardy, A. R. et al. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* **105**, 3552-60 (2005).

31. Savi, P. et al. The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. *Proc Natl Acad Sci U S A* **103**, 11069-74 (2006).

32. 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).

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

34. Brass, S. Cardiovascular biology. Small cells, big issues. *Nature* **409**, 145, 147 (2001).

35. Herbert, J. M. & Savi, P. P2Y12, a new platelet ADP receptor, target of clopidogrel. *Semin Vasc Med* **3**, 113-22 (2003).

36. 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).

CHAPTER 2

G_i-COUPLED PURINERGIC RECEPTORS ON MICROGLIA

G_i-COUPLED PURINERGIC RECEPTORS ON MICROGLIA

The G_i-coupled $P2Y_{12}$ receptor is expressed on brain microglia but not peripheral or infiltrating macrophages

We, and others, have previously shown that $P2Y_{12}$ transcripts are expressed by glial cells of the spinal cord and brain, specifically microglia[1,2]. 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, including enhanced green fluorescent protein (GFP) expressed from the CX_3CR1 fractalkine receptor promoter[3] and the integrin CD11b (**Fig. 1a, b**). Moreover, $P2Y_{12}$ immunoreactivity was not observed in GFAP-expressing astrocytes (**Fig. 1a**). Higher magnification images showed that $P2Y_{12}$ protein was localized predominantly to the cell surface, including the elaborate ramified processes emanating from the cell body (**Fig. 1c**). 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 receptor immunoreactivity (**Fig. 1a**), but showed normal prevalence, distribution, and

morphology of "resting" microglia (**Fig. 2**), suggesting that the receptor is not required for proper development or CNS localization of these cells.

Few markers exist that can differentiate between microglia of the central nervous system and macrophages that reside in peripheral tissues or infiltrate the CNS after injury. Previous *in situ* hybridization studies have shown that $P2Y_{12}$ transcripts are observed in microglia but not spleenic macrophages[1]. Here, we confirm this observation at the protein level by showing that peripheral macrophages are devoid of $P2Y_{12}$ immunoreactivity (**Fig. 1b**), demonstrating that this receptor is, indeed, an excellent molecular marker for differentiating between these two closely related phagocytic cell types.

$P2Y_{12}$ is down regulated upon microglial activation

Microglia are believed to mediate a number of physiological responses to neural injury that include both short and long term actions[4]. Expression of various cell surface markers and cellular phenotypes are known to change following CNS injury[5] and we therefore asked whether $P2Y_{12}$ expression varies with the extent of microglial activation. To address this question, we first used an *ex vivo* tissue preparation to examine the time-course of $P2Y_{12}$ expression following neural injury. To induce neural injury and facilitate microscopic examination over an extended period, thick (400 μm) tissue slices were cut from hippocampi of *Cx3cr1*$^{+/GFP}$ expressing neonatal (P4–7) mice. Over a 24 h time-course, we observed trauma-induced activation of microglia as typically characterized by

their transformation from a highly ramified to amoeboid morphology[6]. Interestingly, $P2Y_{12}$ expression showed a dramatic and continuous decrease such that by 24 h, expression was barely observable (**Fig. 3a**). This correlated with a decrement in the number of primary projections emanating from the cell body as microglia progressed towards a more amoeboid state (**Fig. 3b**). Indeed, when $P2Y_{12}$ expression was quantified on a single cell level, a linear correlation was revealed between expression level and the morphological state of the cell (**Fig. 2c-e**).

We next asked whether $P2Y_{12}$ expression changes as a function of microglial activation *in vivo*. To accomplish this, we examined $P2Y_{12}$ immunoreactivity following a bolus injection of lipopolysaccharide (LPS) into the striatum, a treatment that activates microglia within the injected area[7] and which has been shown to decrease 2MeSADP-evoked calcium increases in cultured mouse microglia[8]. Four days after LPS injection, virtually all microglia within proximity of the injection site exhibited an amoeboid-like morphology, and $P2Y_{12}$ immunoreactivity was virtually undetectable in these cells (**Fig. 4a**). 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. 4b, c**). This was further validated by quantitative 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 (data not shown).

Taken together, our data support a relationship between microglial activation and reduced $P2Y_{12}$ expression in two different experimental systems, and suggest that $P2Y_{12}$ is an excellent molecular marker for visualizing brain microglia in the ramified state. Interestingly, recent studies suggest that $P2Y_{12}$ desensitizes upon platelet activation[9], a phenomenon that may represent a functional correlate of the decreased receptor expression that we observe following microglial activation. Together, these findings suggest possible roles for $P2Y_{12}$ receptors at early rather than late stages of the microglial response to injury.

G_i-coupled purinergic receptors

Like platelets, microglia express a plethora of metabotropic and ionotropic purinergic receptor subtypes such as $P2Y_1$, $P2Y_2$, $P2Y_{12}$, $P2X_4$, and $P2X_7$ [10], suggesting that nucleotides play numerous roles in modulating immune responses to a range of pathophysiological insults of the CNS. For example, pharmacological studies have implicated P2X receptors on microglia in the development of neuropathic pain[11] or the release of neuroprotective or proinflammatory agents, such as tumor necrosis factor-α and interleukin-1β[12,13]. When studying the physiological role of $P2Y_{12}$, it is very important to consider other receptors that are activated by extracellular nucleotides. The presence of multiple receptors with similar pharmacological profiles may make it harder to determine the contribution of the $P2Y_{12}$ receptor to a particular cellular phenotype. Therefore, we decided to examine the pharmacology of a $P2Y_{12}$-

related receptor and determine whether this receptor contributes to microglial physiology.

In 2001 when the sequence of $P2Y_{12}$ was discovered[2], it was the only G_i-coupled purinergic receptor known to exist and actually was quite distantly related to the other known P2Y receptors ($P2Y_1$, $P2Y_4$, and $P2Y_6$ which couples to phospholipase C pathways, and $P2Y_{11}$ which activates the synthesis of cAMP). However, in recent years there have been a handful of other purported G_i-coupled P2Y receptors found to lie just adjacent to the $P2Y_{12}$ locus on chromosome 3, and thus two distinct classes of P2Y receptors were classified[14]. This second class of receptors includes $P2Y_{12}$, $P2Y_{13}$, GPR 87, $P2Y_{14}$ (the UDP-glucose receptor), and GPR 171 located within 114 kb of each other. While little is known about the orphan receptors GPR 87 and GPR 171, it is thought that $P2Y_{14}$ is involved in UDP-glucose induced migration of premature hematopoietic stem cells[15,16]. A homology tree and the locus map for this class of receptors are shown in **Fig. 5**. The close proximity of these genes and striking similarity in sequence homology suggests that these receptors are the product of a recent evolutionary gene duplication event. Examining differences in pharmacology and sequence of these related proteins may help elucidate mechanisms of ligand activation.

$P2Y_{13}$ is located only 4.5 kb away from $P2Y_{12}$ on chromosome 3 and shares 48% sequence homology[17,18]. $P2Y_{13}$ is considered to be G_i-coupled because

pertussis toxin blocks ADP inhibition of cAMP production in CHO cells[17]. $P2Y_{12}$ and $P2Y_{13}$ share very similar pharmacological profiles; ADP acts as an agonist, ATP a partial agonist, and AR-C69931MX an antagonist[14]. We further examined the pharmacology of these receptors by two-electrode voltage clamp electrophysiology in *Xenopus laevis* oocytes. $P2Y_{12}$ or $P2Y_{13}$ mRNA was injected into oocytes along with the inwardly rectifying potassium channels Kir3.1 and Kir3.4, which allow K^+ ions to pass through the membrane when activated by the G-protein. As shown in **Fig. 6a**, $P2Y_{12}$ and $P2Y_{13}$ are equally sensitive to ADP. Interestingly, 2MeSADP activates $P2Y_{12}$ at 100 fold less concentration than ADP, whereas 2MeSADP only activates $P2Y_{13}$ roughly 10 fold less concentration than ADP. In addition, the shape of the 2MeSADP-induced $P2Y_{13}$ current is different than the 2MeSADP-induced $P2Y_{12}$ current in that $P2Y_{13}$ inactivation is slower, or 2MeSADP is harder to wash out from $P2Y_{13}$ expressing oocytes. ATP activates both receptors to a similar extent, and ATP is about 50% less potent than ADP at both receptors. Indeed, it has previously been reported that $P2Y_{13}$ in 1321N1 cells bound radioactively labelled 2MeSADP more tightly than $P2Y_{12}$[14]. There are several other differences in the pharmacology of these two receptors as shown in **Fig. 6a**. For example, both ADP-ribose and AP4A are weak agonists to $P2Y_{12}$ but approximately 2-4 fold stronger agonists to $P2Y_{13}$.

Some GPCRs are known to form multimers in their active state[19], so we asked whether there is a synergism of the $P2Y_{12}$ and $P2Y_{13}$ receptors, or if co-expression of the receptors changes the pharmacology. Importantly, co-

expression of both P2Y receptors in ooctyes resulted in an intermediate profile where, for example, 2MeSADP-activated current is greater than the current in $P2Y_{13}$ expressing oocytes but less than that of $P2Y_{12}$ expressing oocytes. In addition, the current inactivation during wash out of 2MeSADP in ooctyes expressing both receptors also shows an intermediate profile. Also, the ADP-induced current is not enhanced when both receptors are expressed together, also suggesting that there is minimal interaction (**Fig. 6b**).

$P2Y_{12}$ is known to be sensitive to inactivation by compounds that modify cysteine residues, such as the active metabolite of the anti-thrombotic drug clopidogrel[20]. Evidence suggests that $P2Y_{13}$ is not able to bind the active metabolite of clopidogrel[14], however the sensitivity of this receptor to thiol reagents is unknown. We applied PCMBS to $P2Y_{12}$ and $P2Y_{13}$ expressing oocytes (**Fig. 6c**), a mercury compound thought to modify cysteine residues in a manner similar to clopidogrel. For these experiments, the mu-opioid receptor (muOR) was co-injected to show that inactivation of P2Y receptors does not result in the inability of the oocyte to be activated by another G_i-coupled receptor. $P2Y_{12}$ expressing oocytes showed complete inhibition of ADP-induced current by 1 mM PCMBS, whereas $P2Y_{13}$ expressing oocytes showed only 30% ADP-induced current inhibition. Since these two receptors share a very similar nucleotide sequence yet display such different sensitivities to thiol reagents, they provide a unique setting to pinpoint the required structural components needed by clopidogrel to inactivate the $P2Y_{12}$ receptor in platelets.

Although its expression profile has not been well defined, $P2Y_{13}$ mRNA transcript was found to be absent from platelets[21] and thus does not contribute to nucleotide-induced thrombosis. However, $P2Y_{13}$ mRNA was reported to be expressed in brain[21] and therefore we were interested to see whether it is expressed in microglia. To address this question, we isolated primary microglia from wild-type neonatal mouse brain, isolated mRNA, and performed PCR with $P2Y_{13}$ specific primers (**Fig. 7a**). Indeed, $P2Y_{13}$ transcripts were apparent in these cells. *In situ* hybridization of adult brain with a $P2Y_{13}$ specific probe (**Fig. 7b**) confirmed low level expression by microglia and showed that no other CNS cell type expressed $P2Y_{13}$ transcripts. Interestingly, it seems that microglia do not express $P2Y_{13}$ protein *in vivo*, since we failed to detect receptor protein using a $P2Y_{13}$ specific antibody (**Fig. 7c**). These results suggest that $P2Y_{13}$ most likely does not contribute significantly to microglial biology *in vivo*.

Due to the close proximity of the $P2Y_{12}$ and $P2Y_{13}$ receptors, we were interested to see if perhaps the $P2Y_{13}$ locus was disturbed in our $P2Y_{12}$–knockout mice. In addition, we were curious to know whether $P2Y_{13}$ transcripts showed the same down-regulation after microglial activation as $P2Y_{12}$. Therefore, we took brains injected with either PBS (vehicle) or LPS to induce inflammation (see above) and performed a northern blot with a $P2Y_{13}$ specific probe. Surprisingly, we found that $P2Y_{13}$ transcripts were down-regulated after LPS injection in a similar manner to $P2Y_{12}$, and knockout animals expressed less transcripts as well as a

second, higher molecular weight species. Therefore, we surmise that the $P2Y_{13}$ locus, although protein is never transcribed *in vivo*, is under the same transcriptional regulatory elements as $P2Y_{12}$. In addition, it is possible that targeted deletion of $P2Y_{12}$ in the knockout animals resulted in an alteration of a $P2Y_{13}$ regulatory element. In any case, it is unlikely that this receptor plays a major role in the biology of these cells due to the fact that receptor protein is apparently never made.

Discussion

A major problem in the field of microglial biology is the inability to detect whether a cell located in close proximity to an injury site is a local CNS microglia or a macrophage that has infiltrated from the periphery. The activated microglia and peripheral macrophage display very similar cellular markers, making it nearly impossible to distinguish between them. Therefore, it is difficult to ascertain the physiological functions of microglia at various stages of activation. $P2Y_{12}$, however, displays a unique expression profile in that it is expressed only by CNS resident microglia and absent from macrophages. In addition, it becomes down-regulated upon microglial activation, making this a very good marker to visualize microglia in the highly ramified, resting state. Also, it may be the best known marker to distinguish activated from non-activated microglia, since most other markers are not completely turned on or off but rather are only up- or down-

regulated. The physiological significance of $P2Y_{12}$ receptor down-regulation is unknown, however it is likely that this receptor functions in the microglial resting state or at a very early time in the activation process.

The discovery of new GPCRs that are closely related to $P2Y_{12}$ and that lie in close proximity on chromosome 3 has elucidate a new class of G_i-coupled purinergic receptors. $P2Y_{13}$ is the most closely related and shares many pharmacological properties. Two important differences in the pharmacological profiles of these two receptors is 1) $P2Y_{13}$ is not activated as strongly by 2MeSADP as $P2Y_{12}$ and 2) $P2Y_{13}$ is not as sensitive to thiol reagents as $P2Y_{12}$, such as the commonly prescribed anti-thrombotic drug clopidogrel. Uncovering the structural requirements for clopidogrel's actions on preventing thrombosis would be very interesting, and these two receptors provide a unique capacity in which to study this mechanism.

Methods

Immunohistochemistry. Anti-$P2Y_{12}$ receptor polyclonal antibody was generated by immunizing rabbits 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 and spleen tissue from adult (3 months) mice was fixed with 4% paraformaldehyde 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 546 or 594, goat anti-rat Alexa 594, or goat anti-mouse Alexa 633 secondary antibodies (Molecular Probes), respectively. Images were taken on a Zeiss Pascal or LSM 510 Meta confocal microscope using 40x oil- or 20x multi-immersion objective, or a Nikon wide-field fluorescent microscope using a 20x lens.

***Ex vivo* hippocampal slice preparation and imaging.** Hippocampal tissue slices (400 μm thick) were prepared from P4–7 mice and stained as described previously[22,23] using anti-$P2Y_{12}$ primary antibody (1:500), and goat anti-rabbit Alexa 594 (Molecular Probes) secondary antibody (1:1000). Slices were stained with anti-$P2Y_{12}$ antisera at 0 h, 8 h, or 24 h after cutting, and imaged on a Zeiss LSM 510 Meta, Leica TCS NT, or Leica SP2 AOBS confocal microscope with a 20x multi immersion or air lens. (n = 3 slices per time-point, ~ 40 cells per slice at the CA3 region.) 15 z-steps spaced 2 μm apart were collected per image (30 μm total depth) and a maximum projection created. $P2Y_{12}$ images were overlayed with GFP images to analyze expression level. To determine

expression level, cell regions were drawn by outlining the cell in the GFP channel, overlaying that region on the $P2Y_{12}$ image, and calculating the mean gray level. Only cells with the entirety of their projections and cell body residing within the slice were quantified. Cellular morphology was determined by counting the number of ramifications projecting immediately from the cell body (primary projections.)

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 d prior to tissue collection. For immuno- and *in situ* hybridization histochemistry, PFA-fixed (20–30 µm 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[24] 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'-GGCTCCCAGTTTA GCATCAC-3'. Quantification of amplified products was carried out as previously described[24].

$P2Y_{13}$ expression analysis. Quantitative RT-PCR, *in situ* hybridization, northern blot and immunohistology were performed as described above. The following primers pairs were used for PCR analysis:

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

5'-CGCTTGGTTCGCCACCTTCTTGTCCCTT-3'

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

5'-GCCAGAAAGAGAGTTGCTTCTTTAGCAATAAACAGC-3'

CX_3CR1: 5'-TTCACGTTCGGTCTGGTGGG-3'

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.

Pharmacology of P2Y receptors by two-electrode voltage clamp electrophysiology. *Xenopus laevis* oocytes were injected with $mP2Y_{12}$ (0.1 ng), $mP2Y_{13}$ (0.1 ng), Kir 3.1 (2 ng), Kir 3.4 (2 ng), or muOR (0.1 ng) mRNA as described[2]. Kir 3.1 and 3.4 were included in all experiments to allow for detection of GPCR-induced flow of K^+ ions across the membrane. The muOR was added as a positive control for inhibition assays. 0.5 ng $P2Y_{12}$ and 0.5 ng $P2Y_{13}$ was used for experiments in which the receptors were coexpressed Three to seven days after injection, electrophysiology recordings were performed using Geneclamp 500 amplifier (Axon Instruments). Cells were clamped –80 mV membrane potential while the recording chamber was perfused with solution containing KCl (70 mM), NaCl (20 mM), $MgCl_2$ (3 mM), HEPES (5 mM), ph 7.4, at room temperature. Ligands (ATP, ADP, ADP-ribose, AP4A, 2MeSADP, damgo, PCMBS, Sigma) were added to the recording solution as indicated.

References

1. Sasaki, Y. et al. Selective expression of Gi/o-coupled ATP receptor P2Y12 in microglia in rat brain. *Glia* **44**, 242-50 (2003).
2. Hollopeter, G. et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**, 202-7 (2001).
3. 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).
4. Streit, W. J. Microglia and the response to brain injury. *Ernst Schering Res Found Workshop*, 11-24 (2002).
5. Streit, W. J., Graeber, M. B. & Kreutzberg, G. W. Functional plasticity of microglia: a review. *Glia* **1**, 301-7 (1988).
6. Stence, N., Waite, M. & Dailey, M. E. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* **33**, 256-66 (2001).
7. 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).
8. Moller, T., Kann, O., Verkhratsky, A. & Kettenmann, H. Activation of mouse microglial cells affects P2 receptor signaling. *Brain Res* **853**, 49-59 (2000).

9. Hardy, A. R. et al. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* **105**, 3552-60 (2005).

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

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

12. 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).

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

14. Marteau, F. et al. Pharmacological characterization of the human P2Y13 receptor. *Mol Pharmacol* **64**, 104-12 (2003).

15. 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).

16. 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).

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

18. Fumagalli, M. et al. Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y(13) receptor. *Biochem Pharmacol* **68**, 113-24 (2004).

19. Milligan, G. G protein-coupled receptor dimerization: function and ligand pharmacology. *Mol Pharmacol* **66**, 1-7 (2004).

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

21. 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).

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

23. Kurpius, D., Wilson, N., Fuller, L., Hoffman, A. & Dailey, M. E. Early activation, motility, and homing of neonatal microglia to injured neurons does not require protein synthesis. *Glia* **54**, 58-70 (2006).

24. 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).

Figure Legends

Figure 1. $P2Y_{12}$ immunoreactivity is localized to the surface of CNS microglia. (**a**) $P2Y_{12}$ antibody (red) was used to visualize receptors in the cortex of adult wild-type (*P2ry12*$^{+/+}$) or $P2Y_{12}$-deficient (*P2ry12*$^{-/-}$) mice. $P2Y_{12}$ immunoreactivity co-localized with GFP fluorescence (green), which was expressed under control of the CX_3CR1 promotor to specifically label microglia in these mice. $P2Y_{12}$ staining was excluded from astrocytes, which were identified by GFAP (blue) expression. No $P2Y_{12}$ immunoreactivity was observed in sections from *P2ry12*$^{-/-}$ mice. Scale bar = 20 μm. (**b**) Staining of spleen and brain sections with anti-$P2Y_{12}$ (red) and anti-CD11b (green) antisera demonstrates absence of $P2Y_{12}$ expression in peripheral macrophages. For spleen, scale bar = 25 μm; for brain scale bar = 20 μm. (**c**) Higher magnification images of brain microglia reveal $P2Y_{12}$ (red) localization to the plasma membrane, whereas soluble GFP (green) is spread throughout the cell body and cytoplasm. Scale bar = 5 μm.

Figure 2. 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 three regions of the brain (striatum, cortex, and hippocampus), retina, and spinal cord in cryostat sections from wild-type (gray) or $P2Y_{12}$-deficient (black) *Cx3cr1* $^{+/GFP}$ mice (n = 2 mice/genotype; 10-30 sections per animal). The number of microglia per 100 μm^2 area is shown (mean ± s.e.m.). (**b**) The area occupied by processes originating from individual microglia

was quantified from whole mount retinas (n = 1 mouse/genotype; 12 sections per animal; mean ± s.e.m.).

Figure 3. Loss of $P2Y_{12}$ expression accompanies microglial transform from highly ramified to amoeboid state. (**a**) Average $P2Y_{12}$ immunoreactivity per microglial cell was measured in hippocampal slices from *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ neonatal mice at 0 h, 8 h, and 24 h after preparation. (**b**) Average number of primary projections per microglial cell was determined at indicated times following hippocampal slice preparation (n = 50-80 cells per time point; values represent mean ± s.e.m.). (**c**) $P2Y_{12}$ expression is linearly correlated with morphological state (number of primary projections) of microglia at 8 h time-point (R^2 = 0.93). Data derive from 3 separate experiments and were normalized to background fluorescence of the slice. (**d**) Analysis of GFP fluorescence (green) and $P2Y_{12}$ immunoreactivity (red) in hippocampal slices from *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ neonatal mice shows complete loss of $P2Y_{12}$ expression by 24 h. Scale bar = 50 µm. White boxes highlight representative microglia, morphology of which is depicted in panels (**e-h**). Scale bar = 15 µm.

Figure 4. Activated microglia lack $P2Y_{12}$ expression *in vivo*. (**a**) Striatal sections from vehicle (PBS)- or lipopolysaccharide (LPS, 5 µg)-injected *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ 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 µm. (**b**) *In situ*

hybridization with $P2Y_{12}$ antisense probe showed a substantial loss of $P2Y_{12}$ mRNA expression following LPS injection. Anti-GFP antibody was used to visualize microglia (green). Scale bar = 20 μm. (**c**) Northern blot analysis showed a decrease in $P2Y_{12}$ mRNA expression in tissue taken from a region surrounding the LPS injection site relative to PBS-injected control. Probe specificity was verified by lack of signal from *P2ry12*$^{-/-}$ tissue. Cyclophilin transcripts were analyzed to verify equivalent sample loading.

Figure 5. Relationship of $P2Y_{12}$ to other purinergic receptors. (**a**) A phylogenetic tree of mammalian P2Y receptors reveals two distinct classes. Class 1 includes G_q-coupled receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and G_s-coupled receptor $P2Y_{11}$. Class 2 includes the purported G_i-coupled receptors $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$, GPR 87, and GPR 171. (**b**) A diagram of *Mus musculus* chromosome 3q24-25 shows the close proximity of $P2Y_{12}$ and the newly discovered G_i-coupled receptors.

Figure 6. Pharmacological profiles of the $P2Y_{12}$ and $P2Y_{13}$ receptors. (**a**) *Xenopus laevis* oocytes expressing the $P2Y_{12}$ or $P2Y_{13}$ receptor (with Kir 3.1 and Kir 3.4 to allow for GPCR-activated ion flow across the membrane) were exposed to Ringer's solution containing the nucleotides indicated, and the resulting change in current was measured by two-electrode voltage clamp electrophysiology. Note the differences in current amplitude after 2MeSADP, ADP-ribose, and AP4A. Scale bar represents 100 s, 200 nA. Oocytes expressings Kirs without GPCRs showed no nucleotide-evoked currents (data

not shown). All oocyte experiments were performed in the presence of Kir 3.1 and Kir 3.4. (**b**) Co-expression of $P2Y_{12}$ and $P2Y_{13}$ receptors results in an intermediate pharmacological profile, suggesting that there is little or no physiological interaction between the receptors. Scale bar represents 100 s, 100 nA. (**c**) $P2Y_{12}$ is more sensitive to inhibition by the thiol reagent PCMBS than $P2Y_{13}$, supporting the observation that the latter receptor is non-responsive to the anti-thrombotic, cysteine modifying drug clopidogrel. $P2Y_{12}$ expressing oocytes showed an inhibition of ADP-induced current after a brief application of PCMBS. However, PCMBS did not inhibit damgo-induced activation of the muOR receptor, another G_i-coupled receptor that was co-injected as a positive control. Interestingly, $P2Y_{13}$ expressing oocytes showed a greatly reduced inhibition of ADP-induced current by PCMBS whereas the muOR activation remained unchanged in these cells. As expected form pervious results, so expression of $P2Y_{12}$ and $P2Y_{13}$ receptors showed an intermediate phenotype, and the absence of either puringeric receptor resulted in a complete loss of ADP-induced current (negative control). Scale bars represent 50 s or 100 s, 50 nA or 200 nA as indicated.

Figure 7. Microglial expression of $P2Y_{13}$. (**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 *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ mice using a $P2Y_{13}$-specific anti-sense probe revealed weak, but detectable signals in GFP-positive microglia. (**c**) Transiently transfected HEK293T cells expressing mouse $P2Y_{13}$ cDNA show immunoreactivity with anti-$P2Y_{13}$ antibody, whereas vector (pcDNA3)-transfected controls do not. Brain sections from *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ mice lacked detectable immunoreactivity when stained with anti-$P2Y_{13}$ antibody. GFP fluorescence shows location of microglia. Scale bars = 50 μm. (**d**) Northern blot analysis of brains from wild-type or $P2Y_{12}$-knockout mice injected with LPS to induce microglial activation or PBS (vehicle) using a $P2Y_{13}$-specific probe. Upon microglial inflammation, $P2Y_{13}$ transcript level decreases in a manner similar to $P2Y_{12}$. Examination of $P2Y_{12}$-knockout animals reveals that $P2Y_{13}$ transcript levels are diminished even in the healthy, non-activated state, and a second, higher molecular weight species is apparent. A cyclophilin-specific probe was used as a loading control.

Figure 1

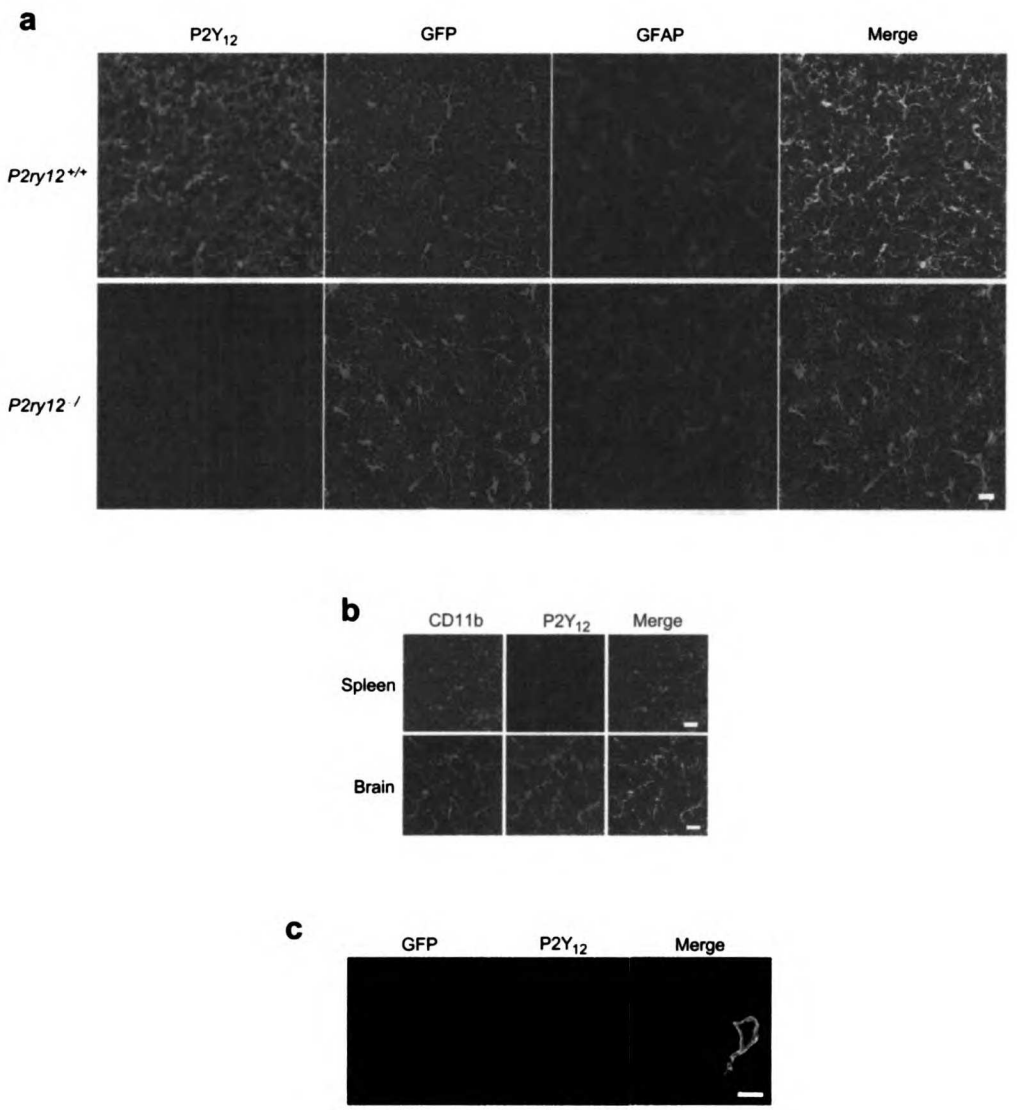
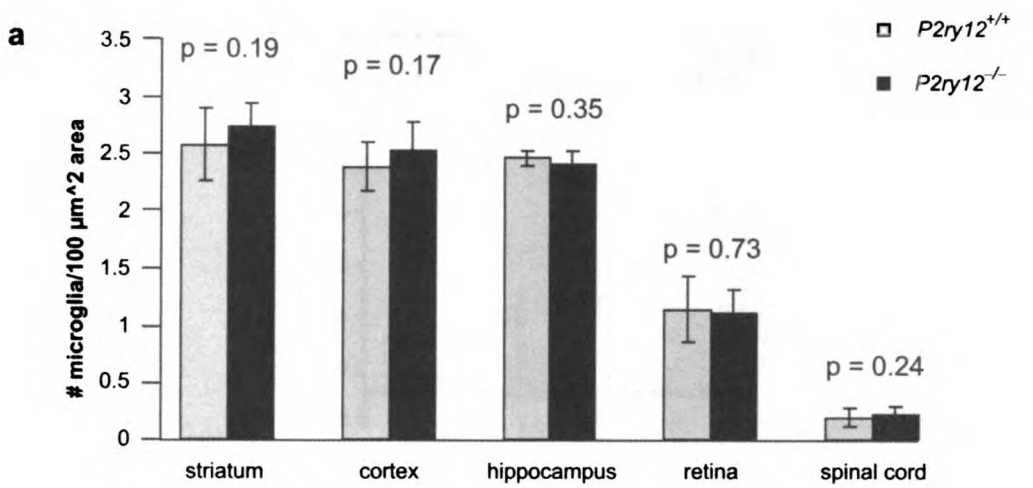

a
P2Y12
GFP
GFAP
Merge
P2ry12+/+
P2ry12-/-
b
CD11b
P2Y12
Merge
Spleen
Brain
c
GFP
P2Y12
Merge

Figure 2


a
P2ry12+/+
P2ry12-/-
microglia/100 µm^2 area
3.5
3
2.5
2
1.5
1
0.5
0
p = 0.19
p = 0.17
p = 0.35
p = 0.73
p = 0.24
striatum
cortex
hippocampus
retina
spinal cord

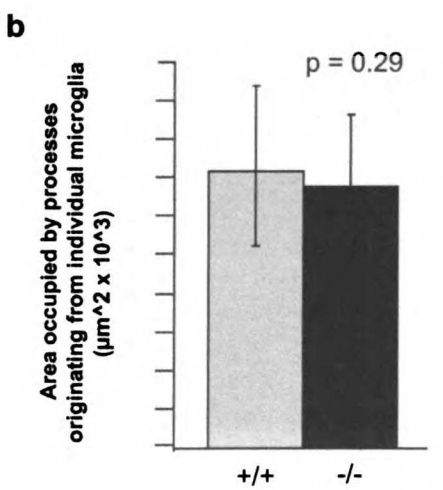

b
Area occupied by processes originating from individual microglia (µm^2 x 10^3)
p = 0.29
+/+
-/-

Figure 3

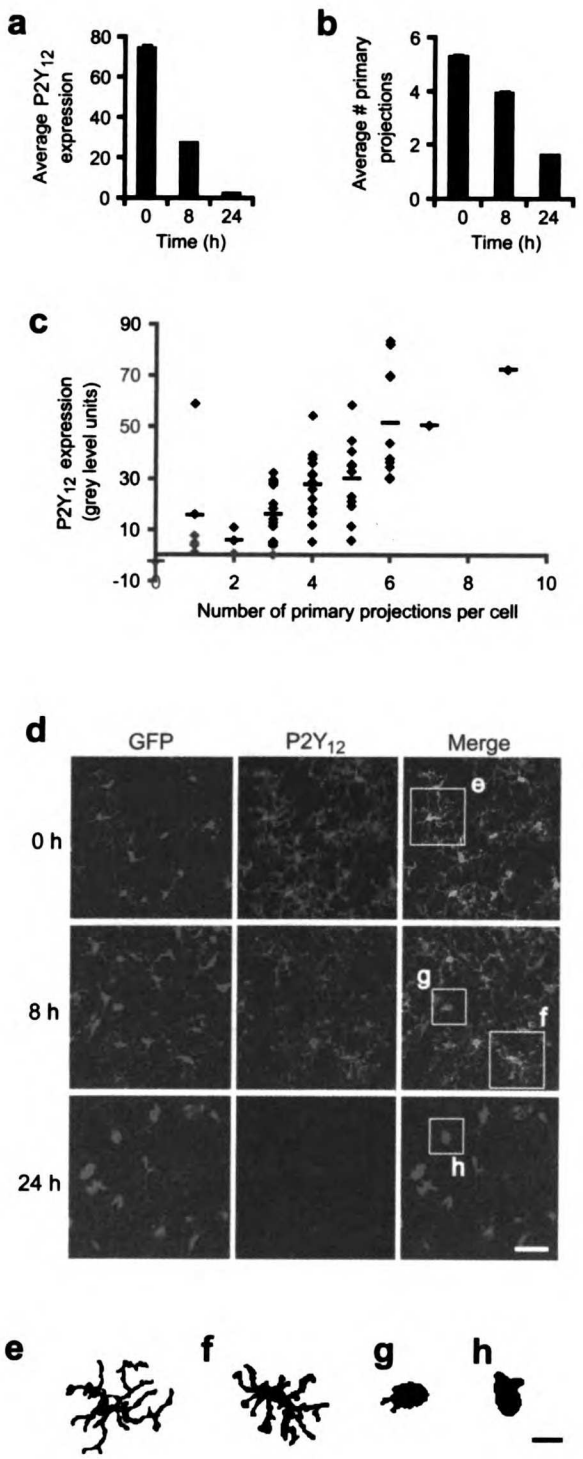

a
Average P2Y12 expression
80
60
40
20
0
0 8 24
Time (h)
b
Average # primary projections
6
4
2
0
0 8 24
Time (h)
c
P2Y12 expression (grey level units)
90
70
50
30
10
-10
0 2 4 6 8 10
Number of primary projections per cell
d
GFP
P2Y12
Merge
0 h
8 h
24 h
e
f
g
h
e
f
g
h

Figure 4

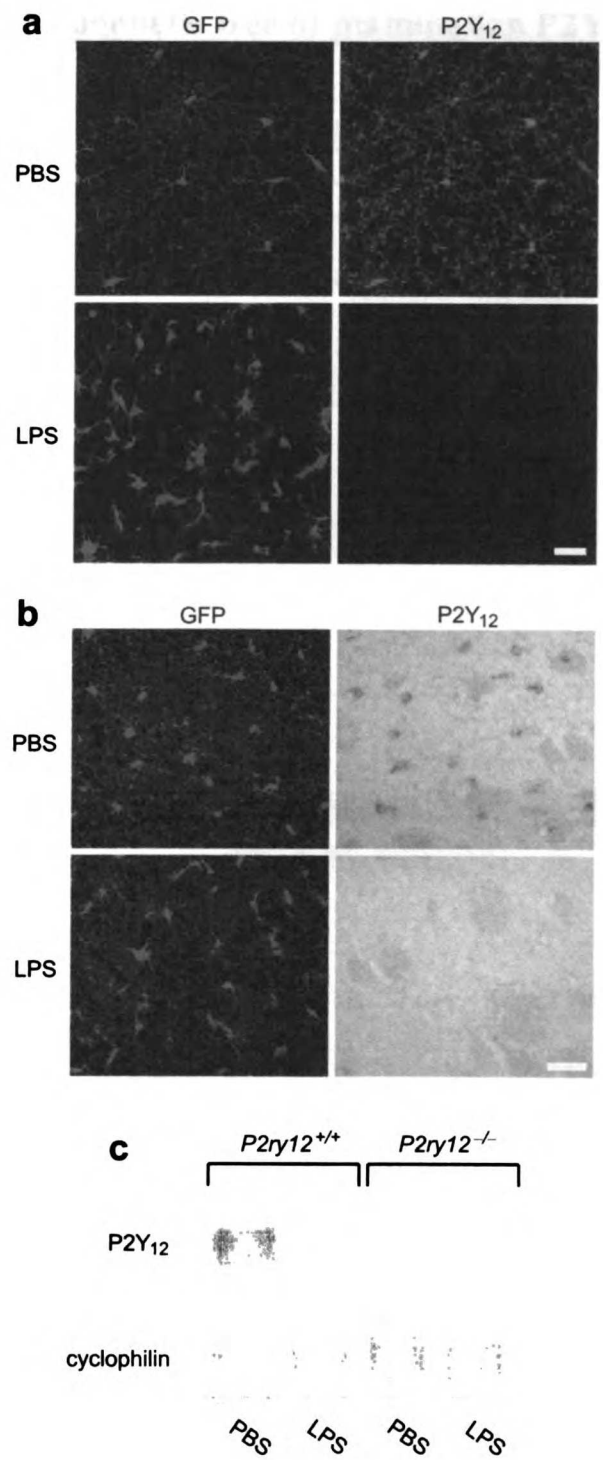
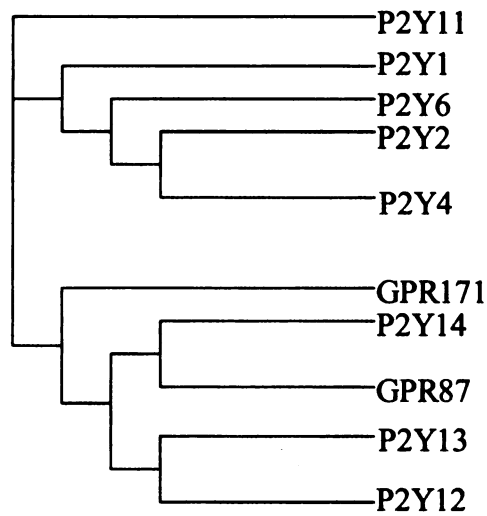

a
GFP
P2Y12
PBS
LPS
b
GFP
P2Y12
PBS
LPS
c
P2ry12+/+
P2ry12−/−
P2Y12
cyclophilin
PBS
LPS
PBS
LPS

Figure 5

a **Phylogenetic tree of mammalian P2Y receptors**



b **Chromosome 3q24-25**

Mus musculus

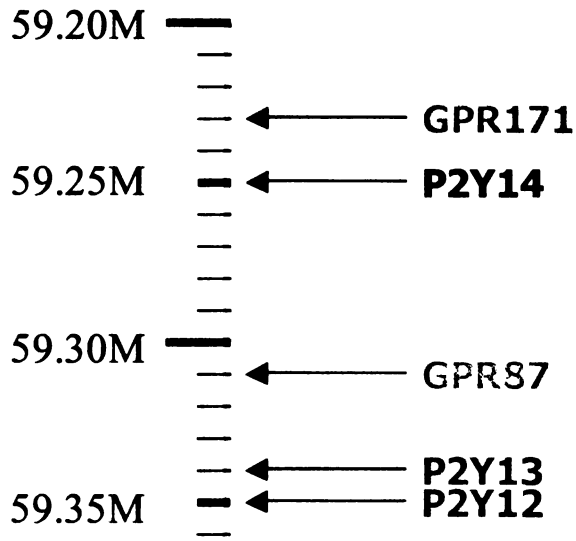


Figure 6

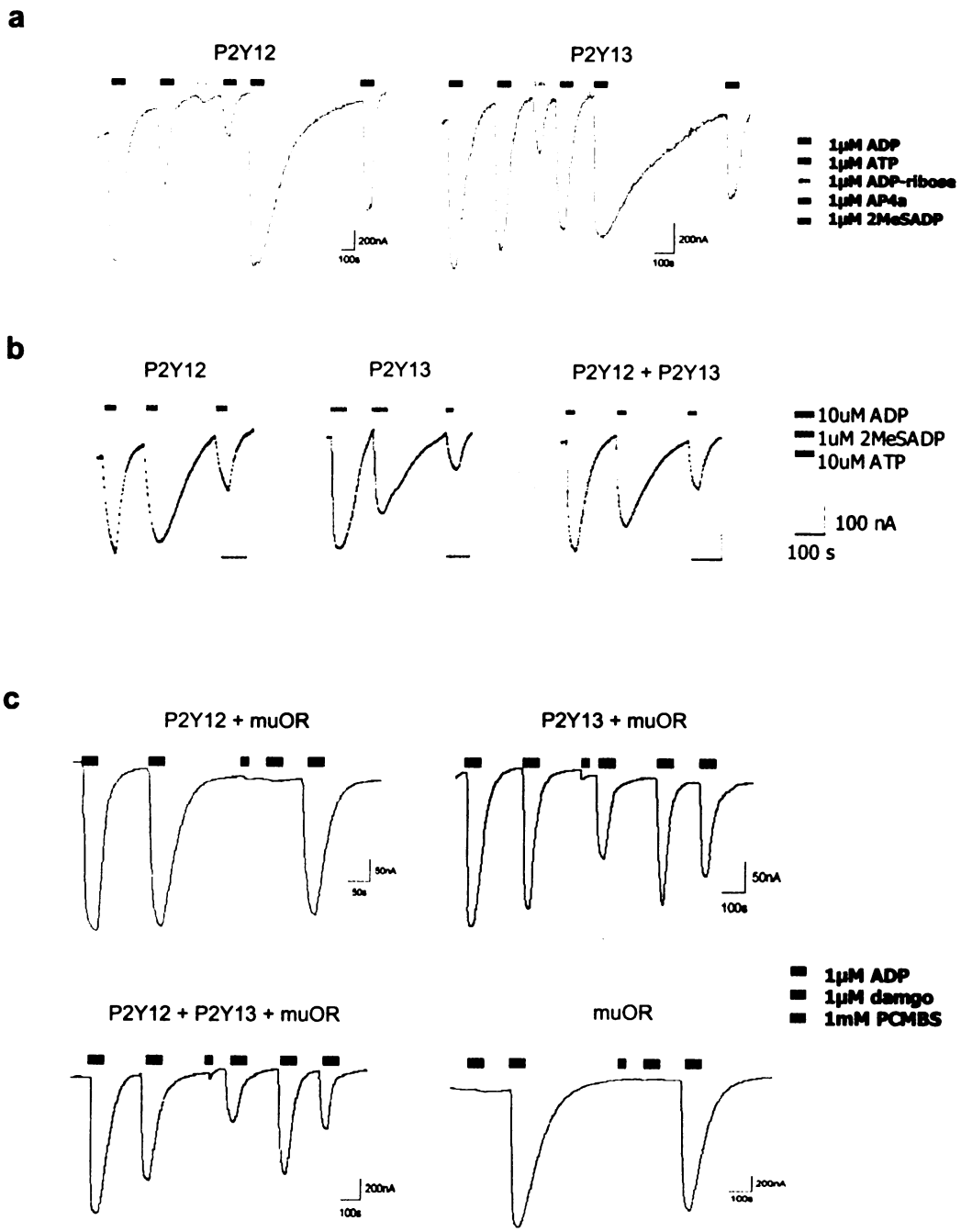
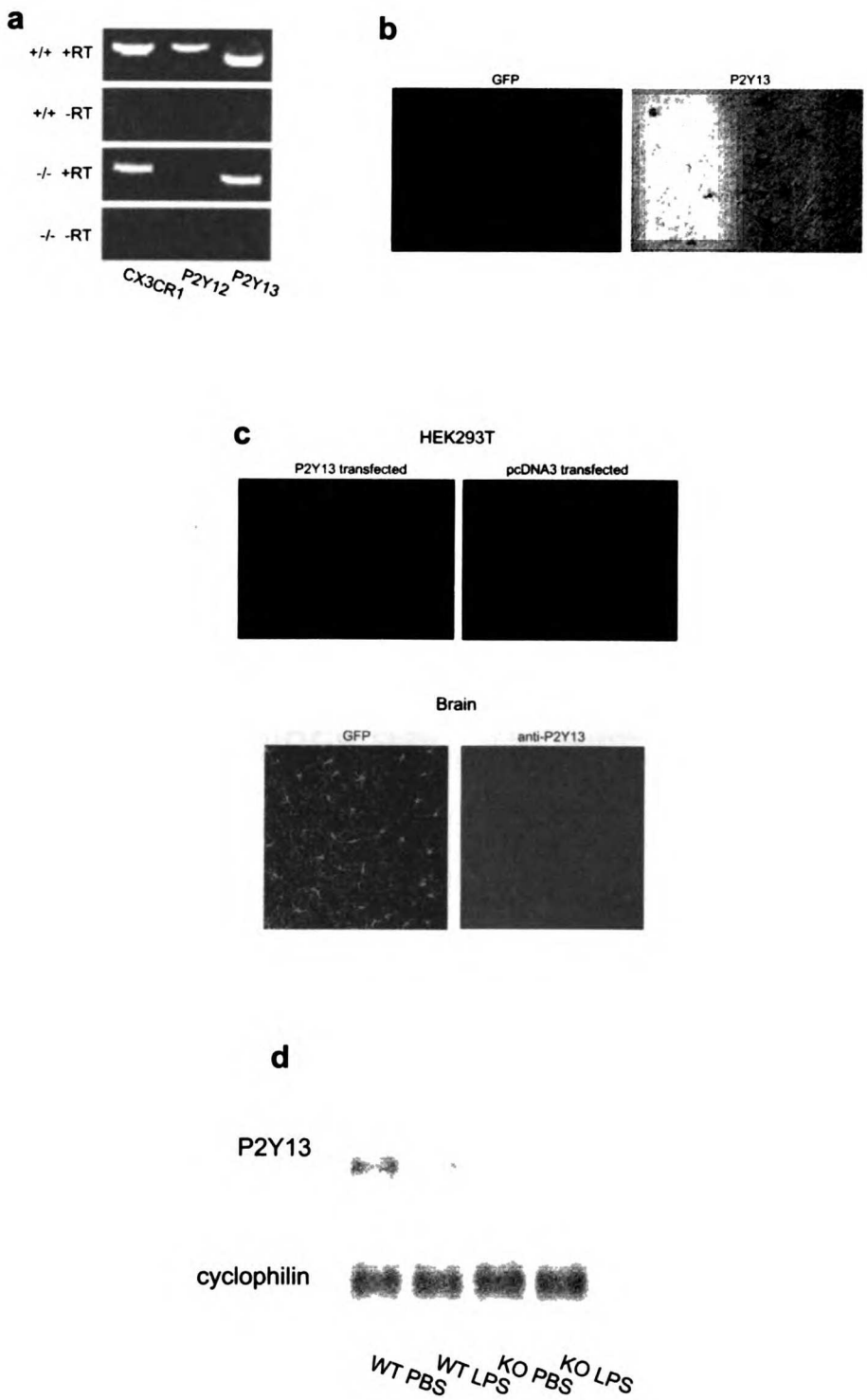

a
P2Y12
P2Y13
200nA
100s
1μM ADP
1μM ATP
1μM ADP-ribose
1μM AP4a
1μM 2MeSADP
b
P2Y12
P2Y13
P2Y12 + P2Y13
10uM ADP
1uM 2MeSADP
10uM ATP
100 nA
100 s
c
P2Y12 + muOR
P2Y13 + muOR
50nA
100s
P2Y12 + P2Y13 + muOR
muOR
200nA
1μM ADP
1μM damgo
1mM PCMBS

Figure 7


a
+/+ +RT
+/+ -RT
-/- +RT
-/- -RT
CX3CR1
P2Y12
P2Y13
b
GFP
P2Y13
c
HEK293T
P2Y13 transfected
pcDNA3 transfected
Brain
GFP
anti-P2Y13
d
P2Y13
cyclophilin
WT PBS
WT LPS
KO PBS
KO LPS

CHAPTER 3

NUCLEOTIDE-EVOKED CHEMOTAXIS OF MICROGLIA REQUIRES $P2Y_{12}$ RECEPTORS

NUCLEOTIDE-EVOKED CHEMOTAXIS OF MICROGLIA REQUIRES $P2Y_{12}$ RECEPTORS

In light of the proposed roles for purinergic receptors in regulating microglial motility, we asked whether $P2Y_{12}$-deficient mice show deficits in cellular responses to exogenously applied nucleotides. Based on our observation that the $P2Y_{12}$ receptor is preferentially expressed in the "resting" state, we used experimental paradigms that allow us to examine cellular responses immediately following nucleotide stimulation. First, we used an *in vitro* culture system to address the potential involvement of $P2Y_{12}$ in ATP/ADP-mediated actin-based lamellipodial 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[1]. 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), consistent with previous observations[2]. In contrast, cells from *P2ry12*$^{-/-}$ mice showed no response to ADP or ATP above that elicited by UTP (50 μM) or vehicle alone (~ 7%). Application of macrophage colony stimulating factor (M-CSF, 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. 1a, b**). Interestingly, pre-incubation with LPS (0.1 mg/ml)

significantly diminished the nucleotide-evoked membrane ruffling of wild-type microglia *in vitro* (**Fig. 2**).

To examine directed motility in response to a localized nucleotide source, we placed purified microglia in a Dunn chemotaxis chamber[3] and observed their behavior in a gradient of ADP or ATP (0 to 50 μM) using phase contrast time-lapse microscopy. Microglia from wild-type mice showed a clear and robust polarization or chemotaxis toward the nucleotide source within the 30 min observation time, whereas $P2Y_{12}$-deficient microglia showed no evidence of membrane ruffling, polarization, or directed movement (**Fig. 1c, d**).

We next asked whether similar phenotypes would be observed when microglia are examined in a more native neuronal environment. To do this, we used the hippocampal slice system described above to monitor microglial chemotactic behavior by time-lapse microscopy following bath application of nucleotides (1 mM ADP). As the nucleotide diffuses into the slice, a gradient is formed which induces microglial chemotaxis towards the periphery of the slice. In brain slices from neonatal (P4–7) wild-type mice, microglia demonstrated robust process extension and cellular migration towards the periphery of the slice that initiated within minutes of nucleotide exposure and was sustained for over 6 h. In contrast, slices prepared from neonatal $P2Y_{12}$-deficient mice show no directed polarization, process extension, or migration in response to the nucleotide stimulus, consistent with our *in vitro* chemotaxis data (**Fig. 3**). In fact, microglia

from mutant mice retract their processes and travel towards the injured neuronal cell body layer of the CA3, consistent with the actions of microglia in wild-type slices in the absence of nucleotides[4].

Finally, we asked whether similar deficits could be observed in the intact living brain of adult mice. We therefore placed a microelectrode containing ATP (20 mM) into the neocortex of *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ or *P2ry12*$^{-/-}$*Cx3cr1*$^{+/GFP}$ adult mice and followed changes in microglial morphology using two-photon time-lapse microscopy. As previously described[5], microglia in adult wild-type animals showed very active extension of cellular processes toward the nucleotide source. In striking contrast, $P2Y_{12}$-deficient mice showed greatly reduced responses during an equivalent 40 min post-injection period, demonstrating that the $P2Y_{12}$ receptor is absolutely required for ATP-mediated process extension of microglia *in vivo* (**Fig. 4**).

Discussion

Taken together, our studies show that the $P2Y_{12}$ receptor is essential for the ability of microglia to respond to extracellular nucleotides by process extension or whole cell movement. Our use of several experimental paradigms revealed that this is true whether microglia are exposed to extracellular nucleotides in the context of a homogeneous culture, or in their native neuronal environment.

Moreover, our data suggest that $P2Y_{12}$ is important in this process at all stages of postnatal development on through adulthood.

Methods

Primary microglia cell culture, membrane ruffling, and chemotaxis assays. Primary microglia were isolated from P1–3 mice using a modified version of the mixed glial culture technique as previously described[6]. Briefly, brains were dissected, homogenized by passing through an 18 G needle, and cultured in DME-H21 with 10% heat-inactivated FBS and penicillin/streptomycin. After 12–14 d, 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 glass chamber slides (Fisher) for membrane ruffling assays or fibronectin-coated coverslips (Becton Dickinson) for chemotaxis assays, washed, and incubated for 4 h in serum-free DME-H21.

Membrane ruffling assays were performed as described (Honda, et. al., 2001) 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 M-CSF (200 ng/ml, BD Biosciences). Microglia were incubated for 5 min at 37° C, fixed with 4% PFA, and stained with rhodamine- or FITC-conjugated phalloidin (Molecular Probes). To determine the effect of LPS pre-incubation on

nucleotide-evoked membrane ruffling, primary microglia were prepared as described and incubated in serum-free media with or without LPS (0.1 mg/ml) for 6 h immediately before a brief (5 min) ATP application (50 μM). Cells were then fixed with 4% PFA and stained with FITC-conjugated phalloidin.

Chemotaxis assays were performed using the Dunn chamber[3] to form a nucleotide gradient from 0 to 50 μM ADP or ATP. 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 μm) was subtracted to obtain final values. Images were processed and analyzed using the National Institutes of Health ImageJ software.

***Ex vivo* hippocampal slice preparation and imaging.** To examine the microglial response to applied nucleotides, acute hippocampal slices were prepared as previously described and incubated in imaging media alone or with 1 mM ADP. Microglia were visualized by GFP fluorescence or by staining with fluorescently tagged isolectin-IB4, and imaged as described[4]. For each image in the time-series, 15 *z*-steps spaced 2 μm apart were collected per image (30 μm total depth) and a maximum projection created for each. Images were taken at 5 min intervals for up to 8 h on Leica TCS NT, or Leica SP2 AOBS confocal microscope with a 20x lens. Image processing and analysis was performed using NIH Image J software.

***In vivo* imaging of microglia.** GFP expressing microglia were imaged by two-photon time-lapse microscopy as described[5,7]. Briefly, ATP (20 mM) was diluted in artificial CSF for intracranial injections. A *z*-stack (~ 100 μm depth, 2 μm *z*-steps) was acquired every 4 min for 40 min. A maximum projection of the 15 *z*-steps centered on the laser ablation or injection site was constructed for each time point (30 μm total depth). Microglial response to ATP injection was determined as described[5]. Changes in GFP fluorescence were monitored as processes entered a circular zone of 70 μm in diameter centered over the injection needle and normalized to an outer region 136 μm in diameter.

References

1. 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).
2. 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).
3. 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).
4. Kurpius, D., Wilson, N., Fuller, L., Hoffman, A. & Dailey, M. E. Early activation, motility, and homing of neonatal microglia to injured neurons does not require protein synthesis. *Glia* **54**, 58-70 (2006).
5. Davalos, D. et al. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* **8**, 752-8 (2005).
6. Giulian, D. & Baker, T. J. Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci* **6**, 2163-78 (1986).
7. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314-8 (2005).

Figure Legends

Figure 1. Nucleotide-evoked membrane ruffling and chemotaxis are abolished in cultured microglia lacking $P2Y_{12}$ receptors. (**a**) Primary microglia from wild-type (*P2ry12*$^{+/+}$) or $P2Y_{12}$-deficient (*P2ry12*$^{-/-}$) mouse brains were exposed to vehicle (DMEH21), ADP (50 μM) or macrophage-colony stimulating factor (M-CSF, 200 ng/ml). Membrane ruffling was visualized within 5 min by staining with rhodamine-phalloidin (red) to detect actin polymerization (white arrowheads). Scale bar = 20 μm. (**b**) Fraction of microglia undergoing membrane ruffling in the presence of vehicle (DMEH21), UTP (50 μM), ADP (50 μM), ATP (50 μM), 2MeSADP (5 μM), and M-CSF (200 ng/ml). Asterisks indicate $P < 0.001$; $n \geq 9$ trials per genotype for each experimental condition, $n \geq 150$ cells per trial; 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 to 0 μM) using a Dunn chemotaxis chamber and visualized by phase contrast time-lapse microscopy. Microglia from wild-type mice showed robust chemotaxis toward the nucleotide source (white arrowheads 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 μm. (**d**) Scatter plot indicates distance (μm) travelled by the leading edge of wild-type (gray) 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.

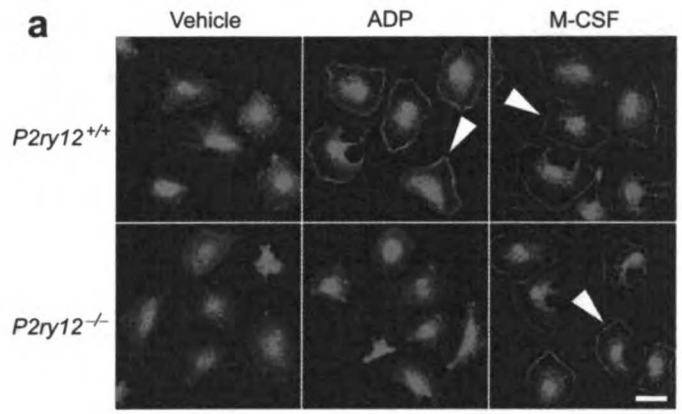
Figure 2. Pre-treatment with LPS diminishes ATP-induced microglial ruffling. Microglia were cultured from brains of wild-type mice and exposed to vehicle (media) or LPS (0.1 mg/ml) for 6 h in serum-free media followed by a 4 h starvation period. Cells were subsequently exposed to ATP (50 μM) for 5 min and membrane ruffling assessed by staining with FITC-conjugated phalloidin (see Methods). Representative images (**a**) and quantification (**b**) illustrates significant decrease in nucleotide responsiveness following LPS exposure. Scale bar = 20 μm. (n = 4 trials per experimental condition, 200-400 cells per trial, $^{*}P <$ 0.01)

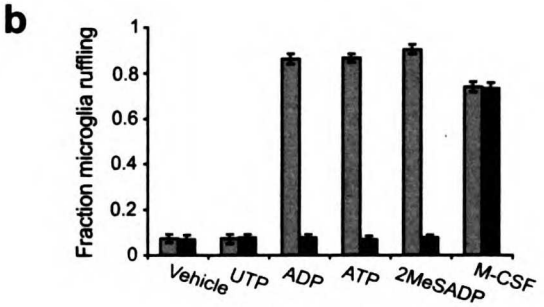
Figure 3. $P2Y_{12}$-deficient microglia do not respond to exogenous nucleotides in brain slices. (**a**) Microglia in hippocampal slices from neonatal (P4–7) GFP-expressing mice were examined by time-lapse confocal imaging. *P2ry12*$^{+/+}$ microglia displayed robust process extension (red arrowheads) and whole-cell locomotion (yellow arrowheads) towards the periphery of the slice (right side of each frame) at various time points following addition of ADP (1 mM) to the bath. Slices from *P2ry12*$^{-/-}$ mice showed no such response over 6 h; green arrowheads show microglial locomotion away from the nucleotide source and towards the CA3 pyramidal cell body layer in *P2ry12*$^{-/-}$slice. (**b**) Microglial branch length (μm) in wild-type and $P2Y_{12}$-deficient slices was measured at each time-point indicated in the presence of 1 mM ADP (n = 5 branches/cell). Positive length change indicates extension, whereas negative length change indicates retraction. Note that all wild-type branches extend, whereas all $P2Y_{12}$-deficient

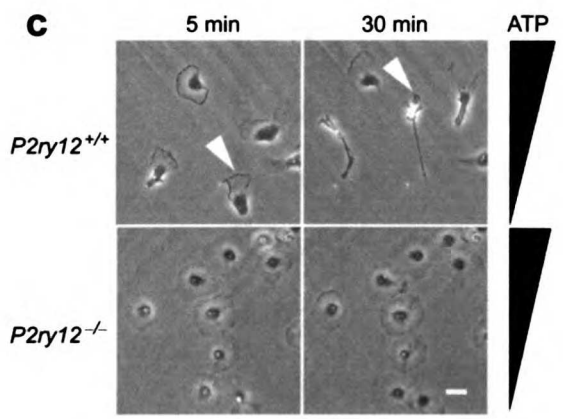
branches retract within the time indicated. (**c**) Microglial locomotion in wild-type and $P2Y_{12}$-deficient slices in the presence (with ADP) or absence of nucleotides. Distance travelled (μm) indicates net cell body displacement towards (positive values) or away from (negative values) the nucleotide source. In the absence of exogenous nucleotides, wild-type or $P2Y_{12}$-deficient microglia migrate toward the pyramidal region, presumably in response to neuronal injury within this cell body layer. Twenty cells residing in the *stratum pyramidale* and *oriens* of the CA3 area of the hippocampus were examined over a 30 min period.

Figure 4. Loss of $P2Y_{12}$ receptors abrogates response of microglia to ATP injection in the living brain. (**a**) Microglia from wild-type (*P2ry12*$^{+/+}$) or $P2Y_{12}$-deficient (*P2ry12*$^{-/-}$) 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 the cortex of wild-type, but not mutant mice. Scale bar = 20 μm. (**b**) Quantification of ATP-evoked process extension in wild-type (grey) and $P2Y_{12}$-deficient (black) brains over a 40 min time course (n = 4 mice per genotype). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's t-test. Values represent mean ± s.e.m.

Figure 1


a
Vehicle
ADP
M-CSF
P2ry12+/+
P2ry12−/−


b
Fraction microglia ruffling
1
0.8
0.6
0.4
0.2
0
Vehicle
UTP
ADP
ATP
2MeSADP
M-CSF


c
5 min
30 min
ATP
P2ry12+/+
P2ry12−/−

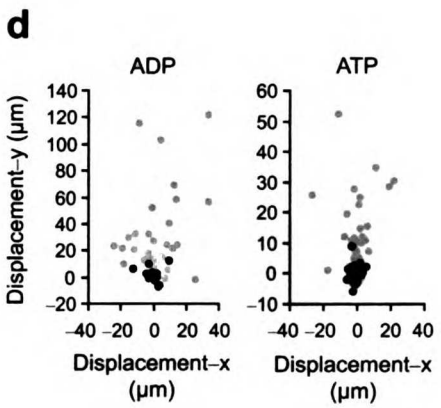

d
ADP
ATP
Displacement-y (μm)
140
120
100
80
60
40
20
0
−20
60
50
40
30
20
10
0
−10
−40 −20 0 20 40
Displacement-x (μm)

Figure 2

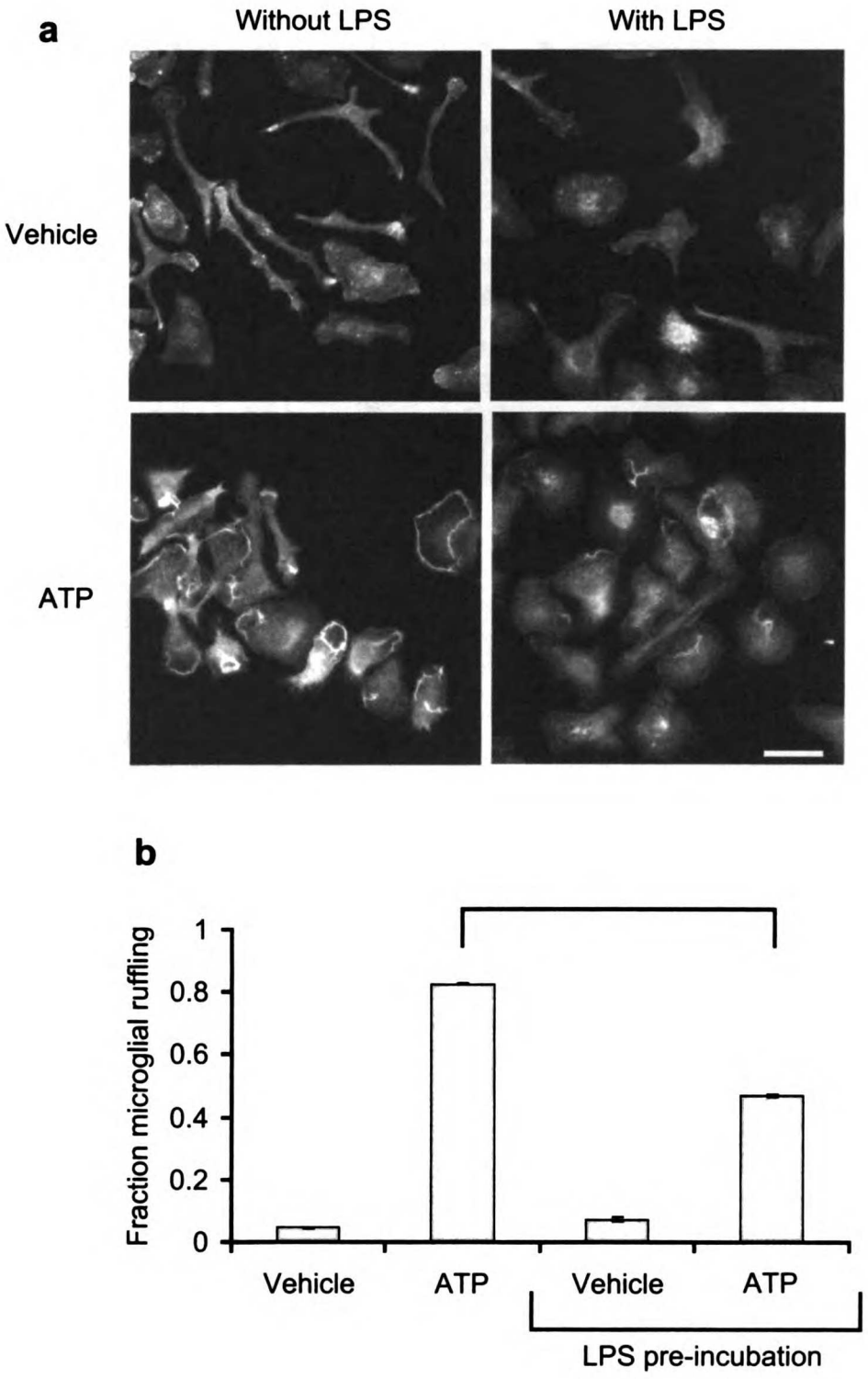

a
Without LPS
With LPS
Vehicle
ATP
b
Fraction microglial ruffling
1
0.8
0.6
0.4
0.2
0
Vehicle
ATP
Vehicle
ATP
LPS pre-incubation

Figure 3

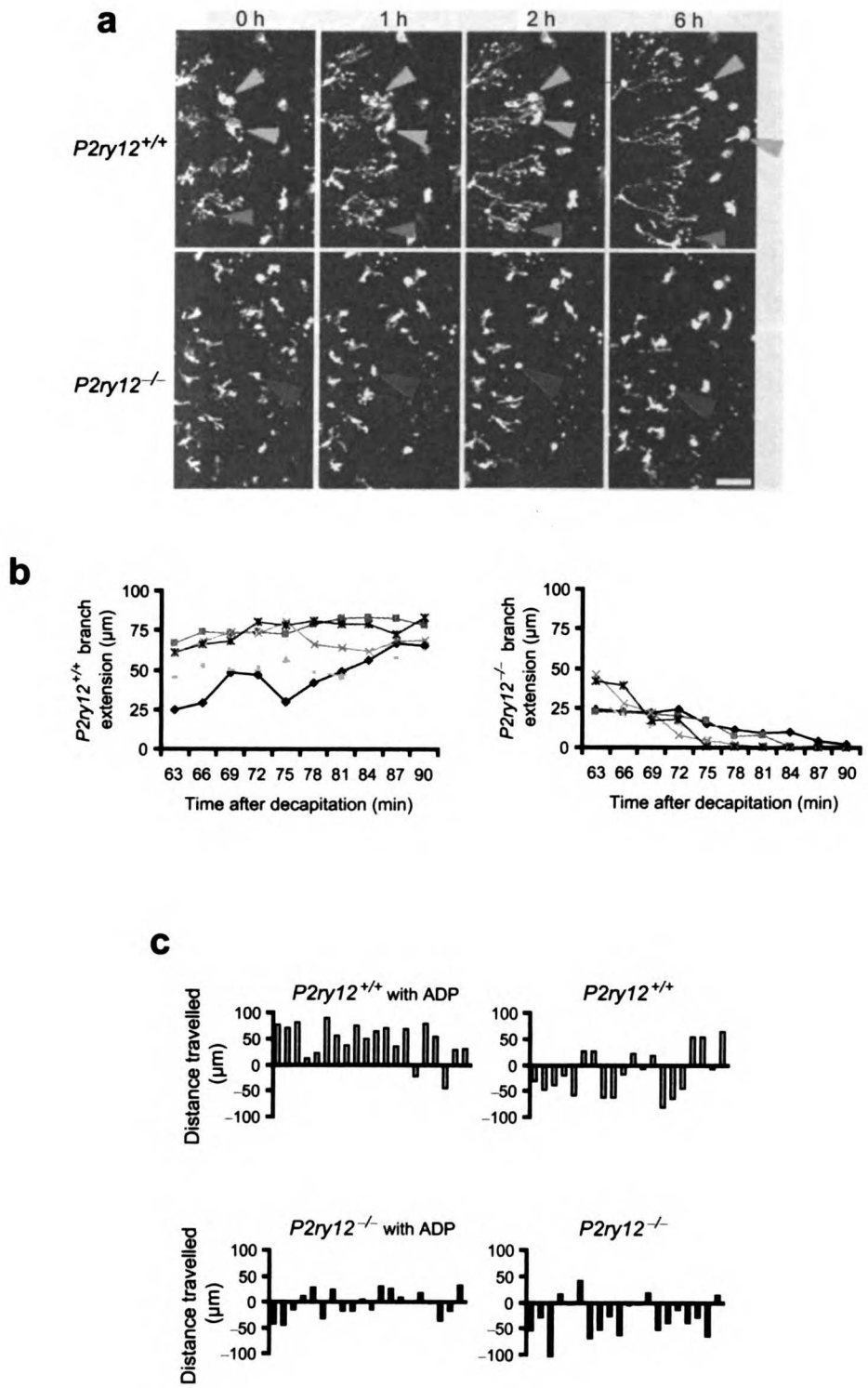
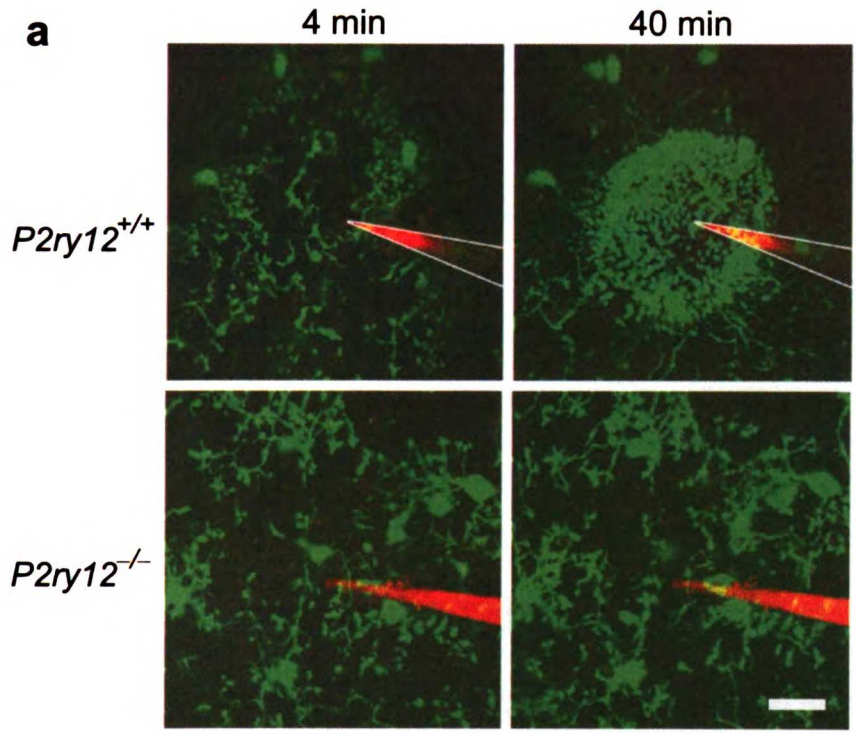
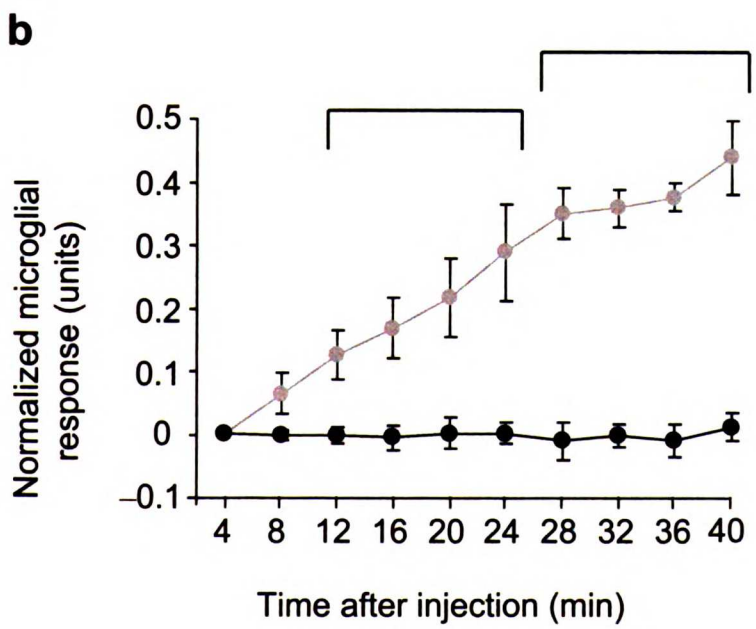

a
0 h
1 h
2 h
6 h
P2ry12+/+
P2ry12−/−
b
P2ry12+/+ branch extension (µm)
P2ry12−/− branch extension (µm)
100
75
50
25
0
63 66 69 72 75 78 81 84 87 90
Time after decapitation (min)
c
P2ry12+/+ with ADP
P2ry12+/+
P2ry12−/− with ADP
P2ry12−/−
Distance travelled (µm)
100
50
0
−50
−100

Figure 4


a
4 min
40 min
P2ry12+/+
P2ry12−/−


b
Normalized microglial response (units)
0.5
0.4
0.3
0.2
0.1
0
−0.1
4
8
12
16
20
24
28
32
36
40
Time after injection (min)

CHAPTER 4

$P2Y_{12}$ MEDIATES THE EARLY STAGES OF MICROGLIAL MORPHOLOGICAL RESPONSE TO TISSUE INJURY

$P2Y_{12}$ MEDIATES THE EARLY STAGES OF MICROGLIAL MORPHOLOGICAL RESPONSE TO TISSUE INJURY

Role of $P2Y_{12}$ in microglial response to cortical laser-ablation

Tissue damage and loss of cellular integrity can lead to the release of nucleotides and other intracellular factors[1], one or more of which may serve to activate nearby microglia. In light of the robust phenotype that we have observed in response to exogenous nucleotides, we asked whether $P2Y_{12}$ is also required for the ability of microglia to rapidly detect and respond to tissue injury[2,3]. We therefore utilized the *in vivo* imaging technique previously described to visualize the response of GFP expressing microglia to localized phototoxicity induced by the two-photon laser. Similar to what we observed with ATP injection, microglia from wild-type mice showed immediate and robust responses characterized by the extension of branches toward the site of damage, whereas *P2ry12*$^{-/-}$ mice showed dramatically reduced chemotactic responses over an equivalent 40 min period. Interestingly, when microglia from mutant mice were examined at 2 h post-injury, the degree of process extension into the damaged area approached that observed with wild-type controls at the 32 min time-point (**Fig. 1a, b**), demonstrating that lack of $P2Y_{12}$ receptors significantly delays, but does not abolish the ability of microglia to respond to local tissue damage.

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]. It has been proposed that this response is mediated by a purinergic receptor because it can be abrogated by apyrase or a high concentration of extracellular nucleotides[2]. To ask whether this baseline motility requires functional $P2Y_{12}$ receptors, we analyzed absolute length change of randomly selected processes from wild-type or $P2Y_{12}$-deficient microglia. No significant differences were observed between genotypes (**Fig. 1c**), suggesting that $P2Y_{12}$ is involved primarily in mediating responses to cellular injury.

Physiological role of $P2Y_{12}$ in microglial response to injury *in vivo*

Here we have shown that microglia from $P2Y_{12}$-knockout mice do not chemotax or project process extensions towards sites of ATP *in vitro*, *ex vivo*, or *in vivo*, and therefore these mice are a valuable tool to assess the contribution of nucleotides to microglial activation after injury. We have also shown that microglia from $P2Y_{12}$-deficient mice are still able, albeit slowly, to morphologically respond to laser-induced damage in the living brain. However, the contribution of nucleotides to the pathological events that occur following other CNS diseases or injuries has not been characterized. To this aim, we performed various animal models that induce microglial activation in $P2Y_{12}$-deficient mice to determine the physiological significance of this signaling pathway on severity of disease.

When the sciatic nerve of the mouse is lesioned, the primary afferent terminals that project into the spinal cord will undergo degenerative atrophy[4], and microglia in lamina I of the dorsal horn will become activated[5]. As previously mentioned, $P2Y_{12}$ is expressed in platelets where it functions in coagulation after vascular injury, and as expected $P2Y_{12}$–deficient mice display a slight bleeding disorder. This is important to keep in mind while performing animal models because increased bleeding by knockout mice may exacerbate the injury. In this regard, the sciatic nerve injury is ideal in that it allows for the surgery to be performed on peripheral tissue while the affects of the injury can be observed centrally. This surgery can be adapted to induce mechanical allodynia, a model of human peripheral neuropathies such as sciatica, an recent evidence suggests that microglia play a central role in this pain process[5]. In this spared nerve model, the sural and perineal branches of the sciatic nerve are cut just below the knee at the point of branching, while the tibial branch is spared. Microglial activation occurs following both the whole-sciatic nerve and spared nerve model, however the former provides a more robust response.

To determine microglial response to injury, we performed the whole sciatic nerve lesion model on wild-type and $P2Y_{12}$–deficient mice. Since pervious results suggested that $P2Y_{12}$ plays a role at early stages of the microglial response to injury, we decided to look at early time-points of activation. At 1 d post-surgery, microglia began to accumulate around the affected site, and gradually continued to increase over the next few days. At all time-points acquired, there was no

significant difference in the increase of microglial cell density on the ipsilateral side between wild-type and $P2Y_{12}$–deficient animals (**Fig. 2**). To determine whether $P2Y_{12}$–deficient mice display altered mechanical allodynia, we performed the spared nerve model and used a behavioral test[4,5] to look for changes in pain hypersensitivity. At 24 h to 2 weeks post-surgery, there was no significant difference in mechanical allodynia observed between wild-type and $P2Y_{12}$–deficient animals (data not shown), suggesting that this receptor does not play a major role in pain.

We have also looked at $P2Y_{12}$–deficient animals in other models such as stroke[6], glioma[7], stab wound[8], herpes virus infection in the retina[9], and experimental autoimmune encephalomyelitis[10] (an animal model of multiple sclerosis.) In all of the models, we were unable to determine a physiological consequence of receptor loss. However, these animal models offer much lower time-resolution than *in vivo* imaging, so it is possible that a difference in microglial migration towards an injury site simply went unnoticed.

Discussion

Although other molecules such as fractalkine, macrophage colony stimulating factor (m-CSF) or cannabinoids have been implicated in microglial migration[11-13], the specific molecules that mediate morphological responses to injury *in vivo*

remain elusive. Recent studies have suggested that ATP is released at a CNS injury site[14] and may be involved in microglial reponse[2]. Since $P2Y_{12}$-deficient mice show no microglial morphological response to applied ATP in any of the experimental paradigms we tested, we may use these mice to determine the contribution of ATP signaling in injury models.

Our *in vivo* imaging studies have shown that the $P2Y_{12}$ receptor is required for microglia to rapidly respond to laser-induced cortical damage, and other signaling pathways may mediate the later stages of this response. Whole animal injury models have not yet elucidated a physiological role for this receptor in microglial accumulation around an injury site or mechanical allodynia after spared nerve injury. It is possible that the time-resolution of these models is not sensitive enough to pick up a difference in microglial migration to the site of injury, if in fact microglia from $P2Y_{12}$-knockout mice only take ~ 4 fold longer to arrive at the affected site. However, these mice may be a useful tool to help determine whether the immediate microglial response to injury is critical to neuronal survival.

Methods

***In vivo* imaging of microglia.** GFP expressing microglia were imaged by two-photon time-lapse microscopy as described[2]. A *z*-stack (~ 100 μm depth, 2 μm

z-steps) was acquired every 4 min for 40 min. A maximum projection of the 15 *z*-steps centered on the laser ablation was constructed for each time point (30 μm total depth). Changes in GFP fluorescence were monitored as processes entered a circular zone of 35 μm in diameter centered on the ablation and normalized to an outer region of 97 μm in diameter.

Sciatic nerve lesion. The sciatic nerve of wild-type or $P2Y_{12}$-deficient mice were lesioned using a modified protocol as described[4]. In brief, adult mice were anesthetized with isoflurane and placed under a dissecting microscope. The eyes were kept lubricated and a heating lamp was used to maintain body temperature. An incision was made through the skin and muscle of the right hind limb directly over the hollow area behind the knee. The sciatic nerve was located at the point in which it branches to form the perineal, tibial, and sural nerves. A suture was tied around the entire sciatic nerve above the branch point, and a 1 mm section of the nerve was removed below the suture. The muscle was then sutured closed and skin stapled. The mouse was sacrificed at 1, 2 or 3 d pos-surgery for microglial analysis. The animal was perfused with 4% PFA, the spinal cord was removed and sectioned (20 μm) at lumbar region 4, 5, and 6, and microglia were visualized by GFP fluorescence. The total number of microglia in a region of gray matter (within 100 μm from the tip of the dorsal horn) was counted (NIH ImageJ software), and the increase in cells on the ipsilateral versus contralateral side was plotted over time.

References

1. Cook, S. P. & McCleskey, E. W. Cell damage excites nociceptors through release of cytosolic ATP. *Pain* **95**, 41-7 (2002).
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. 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).
5. Tsuda, M. et al. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* **424**, 778-83 (2003).
6. Derugin, N. et al. Magnetic resonance imaging as a surrogate measure for histological sub-chronic endpoint in a neonatal rat stroke model. *Brain Res* **1066**, 49-56 (2005).
7. Fan, Q. W. & Weiss, W. A. Isoform specific inhibitors of PI3 kinase in glioma. *Cell Cycle* **5**, 2301-5 (2006).
8. Hampton, D. W., Rhodes, K. E., Zhao, C., Franklin, R. J. & Fawcett, J. W. The responses of oligodendrocyte precursor cells, astrocytes and microglia to a cortical stab injury, in the brain. *Neuroscience* **127**, 813-20 (2004).

9. Lavail, J. H. et al. Genetic and molecular in vivo analysis of herpes simplex virus assembly in murine visual system neurons. *J Virol* **79**, 11142-50 (2005).

10. Lock, C. et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* **8**, 500-8 (2002).

11. Cardona, A. E. et al. Control of microglial neurotoxicity by the fractalkine receptor. *Nat Neurosci* **9**, 917-24 (2006).

12. Werner, K., Bitsch, A., Bunkowski, S., Hemmerlein, B. & Bruck, W. The relative number of macrophages/microglia expressing macrophage colony-stimulating factor and its receptor decreases in multiple sclerosis lesions. *Glia* **40**, 121-9 (2002).

13. Walter, L. et al. Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci* **23**, 1398-405 (2003).

14. Wang, X. et al. P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med* **10**, 821-7 (2004).

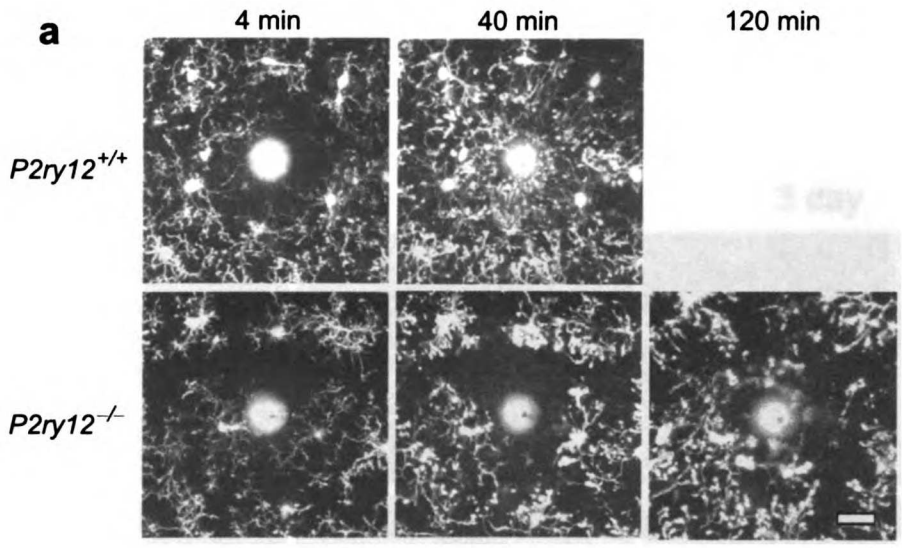
Figure Legends

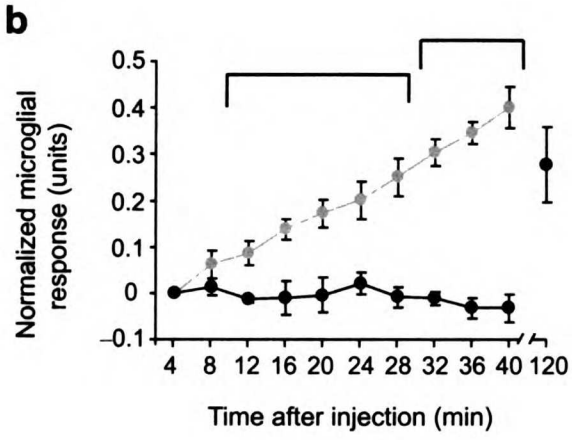
Figure 1. $P2Y_{12}$ receptors mediate the initial response of microglia to focal laser ablation in the living brain. (**a**) Tissue ablation by the two-photon laser (white zone in center) results in microglial process extension towards the site of injury. Microglia from mutant mice showed greatly reduced responses over a 40 min observation period. Scale bar = 20 μm. (**b**) Quantification of process extension toward the site of laser ablation (n = 4 mice per genotype). $P2Y_{12}$-deficient mice were further analyzed at 2 h post-injury (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's t-test. Values represent mean ± s.e.m. (**c**) Microglia from $P2Y_{12}$-deficient animals display normal baseline motility in the living brain. The average processes length change (μm) over a 10 min time scale is displayed on the y-axis (n = 3 animals per genotype, 8-16 processes per animal.)

Figure 2. $P2Y_{12}$–deficient mice display normal microglial response to sciatic nerve lesion. (**a**) The whole sciatic nerve of adult wild-type (WT) or $P2Y_{12}$-knockout (KO) mice was lesioned, and the resulting microglial accumulation in lamina I of the dorsal horn of the spinal cord was imaged by GFP fluorescence. Ipsilateral side is left, contralateral side is right; dorsal horns are down, ventral horns are up in each image. Each section is nicked on the ventral horn of the contralateral side. Number of days post-surgery (1, 2, or 3 d) is indicated. Note the increase in microglial cell density on the ipsilateral side. Scale bar represents 100 μm. (**b**) Quantification of increase in microglial accumulation on the

ipsilateral side over contralateral side after 1, 2, or 3 d post-injury. For 2 d, $P < 0.05$. For 1 and 3 d, $n = 1$ animal, 5-6 sections each; for 2 d, $n = 6$ animals, 5-6 sections each. Wild-type (WT) is represented by blue diamond, $P2Y_{12}$-knockout (KO) Is represented by purple square.

Figure 1


a
4 min
40 min
120 min
P2ry12+/+
P2ry12−/−


b
Normalized microglial response (units)
0.5
0.4
0.3
0.2
0.1
0
−0.1
4 8 12 16 20 24 28 32 36 40 120
Time after injection (min)

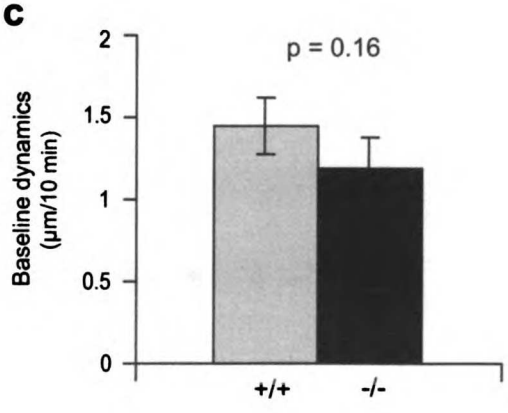
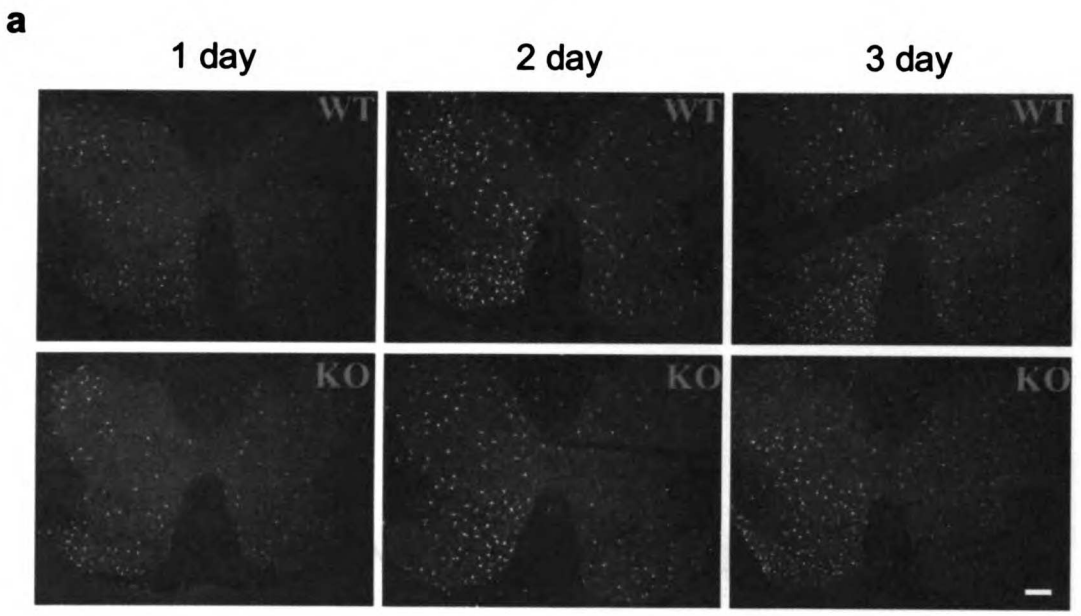
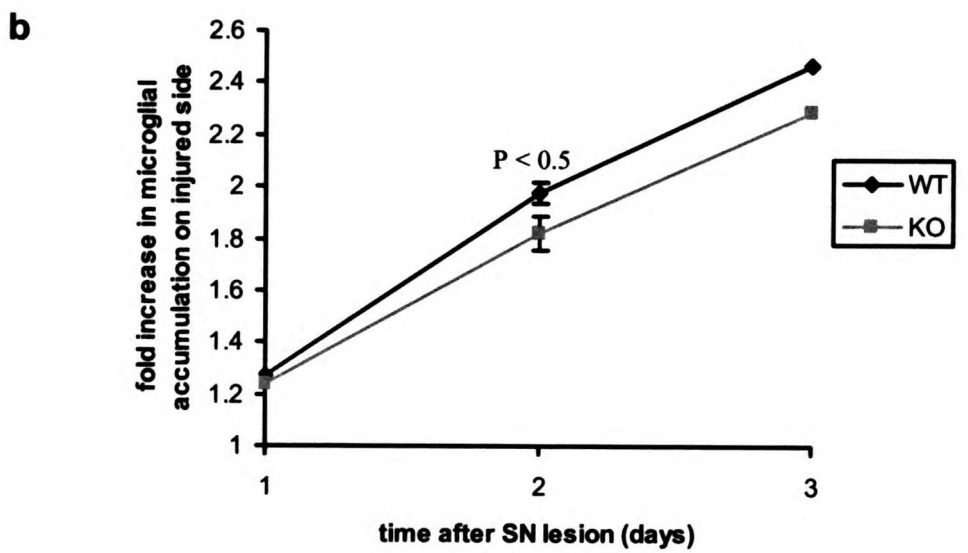

c
p = 0.16
Baseline dynamics (µm/10 min)
2
1.5
1
0.5
0
+/+
-/-

Figure 2


a
1 day
2 day
3 day
WT
WT
WT
KO
KO
KO


b
fold increase in microglial accumulation on injured side
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
P < 0.5
WT
KO
1
2
3
time after SN lesion (days)

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Recent evidence suggests that ATP functions as an important signalling molecule in the microglial response to laser induced damage in the CNS[1]. We now provide genetic proof that nucleotides report tissue injury by directly activating $P2Y_{12}$, a G_i-coupled purinergic receptor on microglia. In this study, three experimental paradigms were used to examine the effects of extracellularly applied nucleotides on microglial behavior, ranging from isolated microglia to intact neural systems. In the most reduced of these, namely primary microglial culture, our analysis showed that applied nucleotides can stimulate migration and elicit morphological changes without contributions from other CNS cell types, such as astroglia or neurons. In the more native environment of the hippocampal slice, where traumatic injury to neural tissue is produced during sample preparation, robust $P2Y_{12}$-dependent polarization and migratory responses to exogenously applied ATP were observed as well, even in the context of endogenously produced injury signals. The most intact of these systems, *in vivo* imaging of the cortex, clearly showed that $P2Y_{12}$ mediates microglial responses to local nucleotide injection in the adult brain. Taken together, these observations suggest that the $P2Y_{12}$ receptor play an important role in modulating microglial response to extracellular nucleotides.

It has recently been reported that inhibitors of connexin channels on astrocytes abrogate microglial response to cortical laser ablation[1], suggesting that astrocytes are activated by ATP and signal to microglia via a different second messenger, or that astrocytes amplify ATP release by positive feedback. In either case, it was unclear whether ATP released from the injury site activates microglia directly, or by promoting the release of other non-nucleotide factors. Our studies suggest that nucleotides have profound effects on microglial polarity and movement through direct activation of $P2Y_{12}$ receptors on these cells, and nucleotides do not initiate the release of other non-nucleotide chemotactic signals. Recent studies have also shown that "resting" microglia are actually quite dynamic and that this activity is dependent on ATP signalling[1,2]. Our results indicate that the $P2Y_{12}$ receptor is not required for this baseline activity, which may therefore be mediated by another purinergic receptor subtype on these cells.

Recent studies have shown that laser induced damage in the cortex of living mice evokes a microglial response that is blocked by broad-spectrum inhibitors of purinergic signalling, apyrase, or a saturating concentration of ATP[1]. Thus, it has been proposed that microglial response to injury involves nucleotide signalling through one or more puringeric receptors. Our results support this hypothesis and demonstrate that the $P2Y_{12}$ receptor plays an important role in this response. While $P2Y_{12}$-deficient mice display a markedly diminished response to focal injury over a 40 min time-period, this deficit becomes less apparent at later time-

points, suggesting that other signalling mechanisms compensate for loss of $P2Y_{12}$ *in vivo*. Other purinergic receptor subtypes may account for such compensation, but signalling by non-nucleotide factors (such as M-CSF, cannabinoids, or fractalkine) seems more likely since $P2Y_{12}$-deficient mice exhibit a complete loss of nucleotide-evoked migration both *in vitro* and *in vivo*. Indeed, this is reminiscent of what is seen in the cardiovascular system, where ADP plays an important role in regulating initial morphological changes in platelets as they transform from an inactive to active state[3-5]. In humans or mice, loss of $P2Y_{12}$ function results in prolonged bleeding times, but platelets can eventually clot since other factors (such as thromboxane A_2) also contribute to the activation process[4]. Despite such functional redundancy, drugs that inhibit platelet $P2Y_{12}$ receptors (such as clopidogrel) have significant cardio-protective effects by decreasing the recurrence of stroke and heart attack, illustrating the importance of this receptor to the overall response to tissue injury.

Microglia express several metabotropic and ionotropic purinergic receptor subtypes[6], suggesting that nucleotides may play various roles in modulating inflammatory responses to a range of CNS injuries or diseases. For example, pharmacological studies have implicated P2X receptors on microglia in the development of neuropathic pain[7] or the release of neuro-modulating agents, such as TNF-α and IL-1β[8,9]. In addition, recent studies have identified a second ADP-activated G_i-coupled receptor, $P2Y_{13}$, with similar pharmacological properties[10] that lies in close proximity to the $P2Y_{12}$ locus. Interestingly, $P2Y_{13}$

was less sensitive to cysteine modifying agents (such as clopidogrel) than $P2Y_{12}$ in electrophysiology assays. As such, analysis of these receptors may uncover the specific structural requirements necessary for clopidogrel's anti-thrombotic actions. While we can detect low levels of $P2Y_{13}$ transcript in microglia, we were unable to observe protein expression *in vivo* using a $P2Y_{13}$-specific antibody. These results, together with our functional studies, suggest that $P2Y_{12}$ is a primary site through which nucleotides mediate the rapid microglial responses that we describe here, although a contribution by $P2Y_{13}$ cannot be formally excluded.

Our findings support the involvement of $P2Y_{12}$ receptors at early stages of the microglial response to neural injury, perhaps resembling their role in platelet activation. In the latter case, the physiological consequences of $P2Y_{12}$ receptor inactivation (genetic or pharmacologic) can be identified through quantitative measurements of blood coagulation rates throughout the course of the clotting process. In the case of microglia, however, long-term physiological consequences of CNS injury and disease are generally assessed over a period of days or weeks, and thus the identification of $P2Y_{12}$-dependent behavioral or anatomical phenotypes may be difficult to define if, indeed, this receptor plays an early, but redundant role in microglial response to injury. Whether and how a delay of microglial response (as observed in *P2ry12*$^{-/-}$ mice) would affect neuronal survival near an injury site has yet to be determined. However, the clinical benefit of $P2Y_{12}$ antagonists in preventing heart attack and stroke

suggests that modulation of this receptor may also have physiological benefit in the context of neural injury and disease.

Future Directions

While our studies have provided evidence that $P2Y_{12}$ receptors function in microglial activation, the physiological consequence of receptor loss is still unknown. In the future, we would like to look more closely at animal models to determine if $P2Y_{12}$-knockout mice display altered cytokine release, clearance of cellular debris, neuronal survival or regeneration, pain sensitivity, or infiltration of other immune cells. Our studies have focused primarily on microglial migration towards the injury site, and not on other aspects of the microglial inflammatory response.

Here we have shown that $P2Y_{12}$ expression is dynamic and down-regulated upon microglial activation, the physiological significance of which we have not yet determined. For example, do microglia decrease receptor expression so that cells that have reached their destination become desensitized to nucleotide signals and do not leave the affected site? In addition, it would be interesting to determine the cellular factors that influence $P2Y_{12}$ expression in the CNS. To address this question, we would like to take activated mircoglia that do not express $P2Y_{12}$ and expose them to the CNS environment by placing them on top of a hippocampal slice or in the presence of homogenized brain. If $P2Y_{12}$ expression is regained, we may then fractionate the material until individual

factors that are sufficient to induce expression are found. In addition, we may take non-derived bone marrow cells (that contain microglial and macrophage precursors) and expose them to CNS factors to determine if a specific molecule(s) is sufficient to induce expression. It may also be interesting to characterize the $P2Y_{12}$ promoter region and look for known transcription factor binding sites or regulatory elements that may modulate protein levels.

The most significant problem facing the field of microglial biology today is the inability to tell these cells apart from other monocyte-derived cells. Microglia and macrophages share strikingly similar molecular profiles, and consequently there are no good markers to distinguish between these two myeloid cell types. $P2Y_{12}$ is the first marker that can easily differentiate between these cells since protein expression is very robust in microglia of the healthy brain but completely absent from peripheral macrophages. In this regard, we may use the $P2Y_{12}$ promoter to drive expression of various genes of interest specifically in microglia of the CNS. For example, we may insert of the lacZ gene into the $P2Y_{12}$ open reading frame and make a mouse knock-in construct for targeted expression *in vivo*. Of course, platelets will also express this reporter gene, but it is very easy to differentiate between microglia and this megakaryocytic cell type.

In this same manner, we may be able to make microglial specific knockout mice of loxP-flanked genes by expressing Cre recombinase under the $P2Y_{12}$ promoter. Previous studies have used a similar approach in which Cre recombinase is

driven off the CD11b promoter, a molecular marker shared by both microglia and macrophages[11], but these studies are unable to differentiate between the affects of gene deletion in these related cell types. Thus, the $P2Y_{12}$ locus provides the solution to a major problem in studying the microglial inflammatory response to injury and diseases of the CNS.

References

1. Davalos, D. et al. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* **8**, 752-8 (2005).
2. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314-8 (2005).
3. Hollopeter, G. et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**, 202-7 (2001).
4. Andre, P. et al. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest* **112**, 398-406 (2003).
5. 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).
6. Inoue, K. Microglial activation by purines and pyrimidines. *Glia* **40**, 156-63 (2002).
7. Tsuda, M. et al. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* **424**, 778-83 (2003).
8. Suzuki, T. et al. Production and release of neuroprotective tumor necrosis factor by P2X7 receptor-activated microglia. *J Neurosci* **24**, 1-7 (2004).

9. 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).

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

11. Boillee, S. et al. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* **312**, 1389-92 (2006).