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

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Microglia are primary immune sentinels of the CNS. Following injury, these cells migrate or extend processes toward sites of tissue damage. CNS injury is accompanied by 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- and 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 toward nucleotides *in vitro* or *in vivo*. Microglia in *P2ry12*$^{-/-}$ mice show significantly diminished directional branch extension toward 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 imply 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.

In the spinal cord and brain, microglia migrate or project cellular processes toward sites of mechanical injury or tissue damage[1–3], where they clear debris and release neurotrophic or neurotoxic agents[4,5]. As such, microglial activation, or lack thereof, has been proposed to influence degenerative and regenerative processes in the brain and spinal cord. Some aspects of microglial response to injury can be mimicked by injection of ATP or attenuated by broad-spectrum inhibitors of purinergic signaling[1], indicating that nucleotides may function as regulators of microglial behavior *in vivo*.

Microglia express an array of ionotropic ($P2X_4$ and $P2X_7$) and metabotropic ($P2Y_1$, $P2Y_2$ and $P2Y_{12}$) purinergic receptors that have been proposed to have important roles in activation, movement or paracrine signaling by these cells[6,7]. For example, activation of the $P2X_4$ receptor has been implicated in the process whereby nerve damage produces pain hypersensitivity (neuropathic pain), a pathway that may involve nucleotide-evoked release of neurotrophins (BDNF) from microglia[8].

One hallmark of myeloid cells is their ability to undergo morphological changes in response to signaling molecules released after injury or disease[9]. In this regard, ATP- or ADP-evoked membrane ruffling and chemotaxis of cultured microglia have been shown to occur through a pertussis toxin–sensitive signaling pathway, thus implicating G_i-coupled receptor(s)–mediated inhibition of cyclic AMP synthesis[10]. Whereas the vast majority of metabotropic P2Y receptors transduce their signals through G_q-phospholipase C pathways[11], the $P2Y_{12}$ subtype is somewhat unique in that it couples to G_i-adenylyl cyclase pathways[12,13], making it an attractive candidate for mediating morphological responses of microglia to extracellular nucleotides.

The $P2Y_{12}$ receptor was initially identified on platelets, where it regulates their conversion from the inactive to active state during the clotting process[12,14,15]. Thus, growth of a nascent clot is dependent on the release of ADP (and other factors such as thromboxane A_2)[16] 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 clopidogrel (Plavix) block platelet activation and reduce the risk of recurrent heart attack or stroke[17]. 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[14].

In light of the importance of this receptor in platelet activation, we asked whether $P2Y_{12}$ might have 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 signaling pathway through which extracellular purines promote directed microglial movement following CNS injury. Moreover, our findings imply that, as in platelets, signaling in microglia through $P2Y_{12}$ is most relevant during early stages of the activation process.

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RESULTS

$P2Y_{12}$ is expressed on microglia but not macrophages

We, and others, have previously shown that $P2Y_{12}$ transcripts are expressed by glial cells of the spinal cord and brain, specifically microglia[7,12]. 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 colocalization with two independent microglial markers, including enhanced green fluorescent protein (GFP) expressed from the CX_3CR1 fractalkine receptor promoter[18] 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. Notably, $P2Y_{12}$-deficient mice were devoid of receptor immunoreactivity (**Fig. 1a**), but showed normal prevalence, distribution and morphology of 'resting' microglia (**Supplementary Fig. 1** online), indicating that the receptor may not be 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 splenic macrophages[7]. 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[19]. Expression of various cell surface markers and cellular phenotypes are known to change following CNS injury[20], 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 (postnatal days (P) 4–7) 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[3]. Notably, $P2Y_{12}$ expression showed a dramatic and continuous decrease, such that by 24 h, expression was barely observable (**Fig. 2a**). This correlated with a decrement in the number of primary projections emanating from the cell body as microglia progressed toward a more amoeboid state (**Fig. 2b**). 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–h**).

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[21] and which has been shown to decrease 2-methylthio-ADP (2MeSADP)-evoked calcium increases in cultured mouse microglia[22]. Four days after LPS injection, virtually all microglia in the proximity of the injection site exhibited an amoeboid morphology, and $P2Y_{12}$ immunoreactivity was virtually undetectable in these cells (**Fig. 3a**). Reduced $P2Y_{12}$ expression was confirmed at the transcriptional level by *in situ* hybridization histochemistry and northern blotting, in which a substantial decrease in messenger RNA levels was observed (**Fig. 3b**,**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 indicate that $P2Y_{12}$ may be an excellent molecular marker

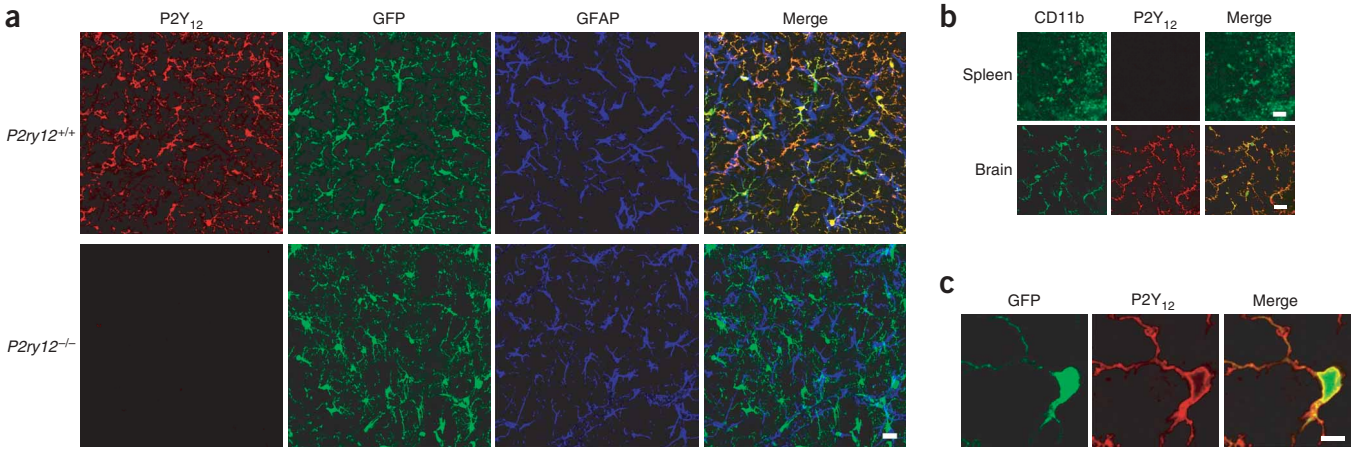

Figure 1 $P2Y_{12}$ immunoreactivity localized to the surface of CNS microglia. (**a**) $P2Y_{12}$ antibody (red) was used to visualize receptors in the cortex of adult wild-type (*P2ry12*$^{+/+}$) or $P2Y_{12}$-deficient (*P2ry12*$^{-/-}$) mice. $P2Y_{12}$ immunoreactivity colocalized with GFP fluorescence (green), which was expressed under control of the CX_3CR1 promoter 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 demonstrating absence of $P2Y_{12}$ expression in peripheral macrophages. For spleen, scale bar is 25 μm; for brain, 20 μm. (**c**) Higher magnification images of brain microglia show $P2Y_{12}$ (red) localization to the plasma membrane, whereas soluble GFP (green) is spread throughout the cell body and cytoplasm. Scale bar, 5 μm.

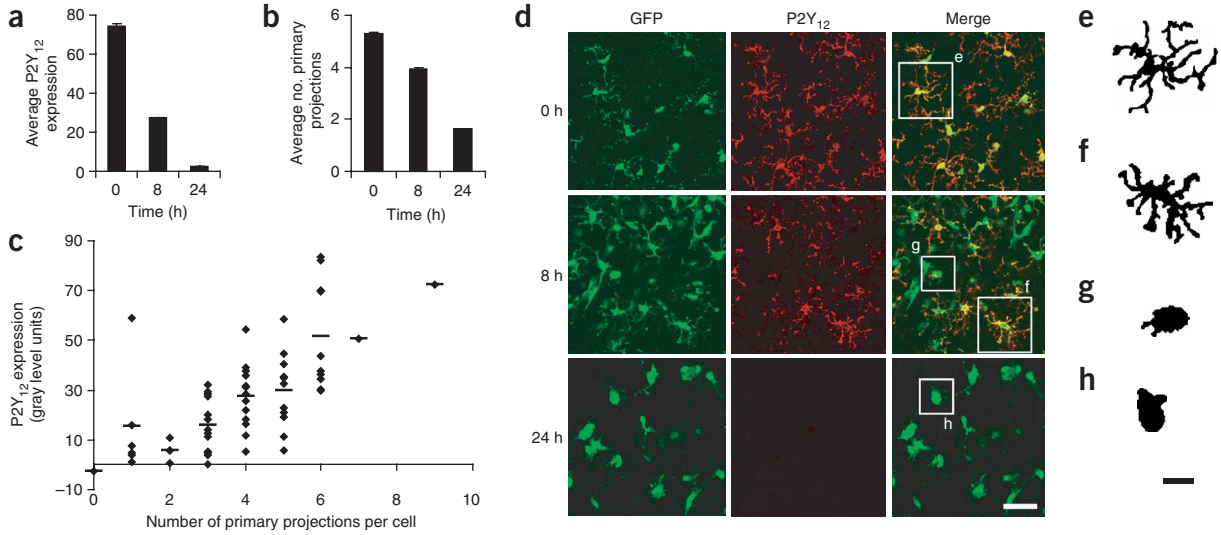

Figure 2 Loss of $P2Y_{12}$ expression accompanies microglial transformation from highly ramified to amoeboid state. (**a**) Average $P2Y_{12}$ immunoreactivity per microglial cell was measured in hippocampal slices from *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ neonatal mice at 0 h, 8 h and 24 h after preparation. (**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; 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 three 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.

for visualizing microglia in the ramified state. Notably, recent studies suggest that $P2Y_{12}$ desensitizes upon platelet activation[23], a phenomenon that may represent a functional correlate of the decreased receptor expression that we observe following microglial activation. Together, these findings indicate possible roles for $P2Y_{12}$ receptors at early rather than late stages of the microglial response to injury.

Nucleotide-evoked chemotaxis 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- and 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[24]. Under these conditions, the vast majority (~87%) of microglia from wild-type mice showed robust membrane ruffling upon application of ADP or ATP (50 μM), consistent with previous observations[10]. 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^{-1}) produced equivalent responses in wild-type and

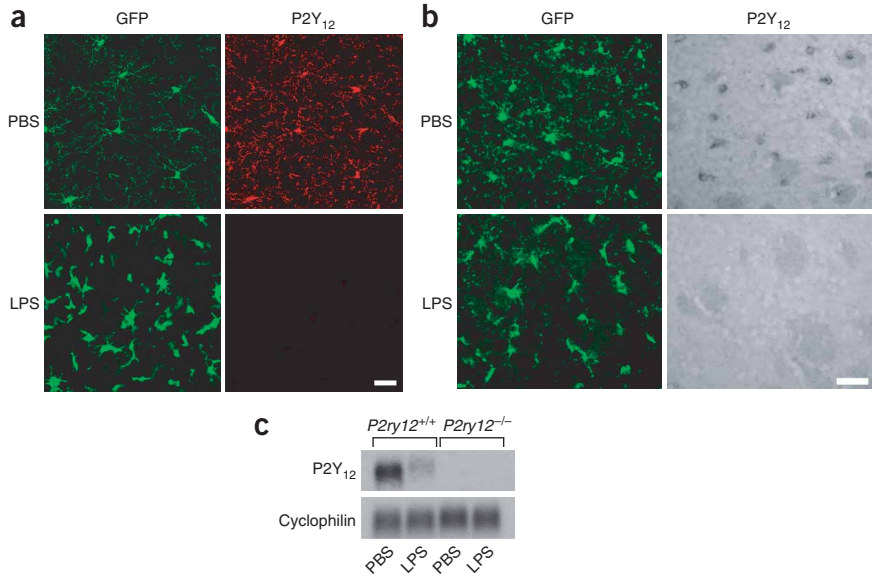

Figure 3 Activated microglia lack $P2Y_{12}$ expression *in vivo*. (**a**) Striatal sections from vehicle (PBS)- or lipopolysaccharide (LPS, 5 μg)-injected *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ mice examined for $P2Y_{12}$ immunoreactivity (red). Note the absence of $P2Y_{12}$ staining 4 d after LPS injection. Similar results were obtained in five 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 vehicle-injected *P2ry12*$^{-/-}$ tissue. Cyclophilin transcripts were analyzed to verify equivalent sample loading.

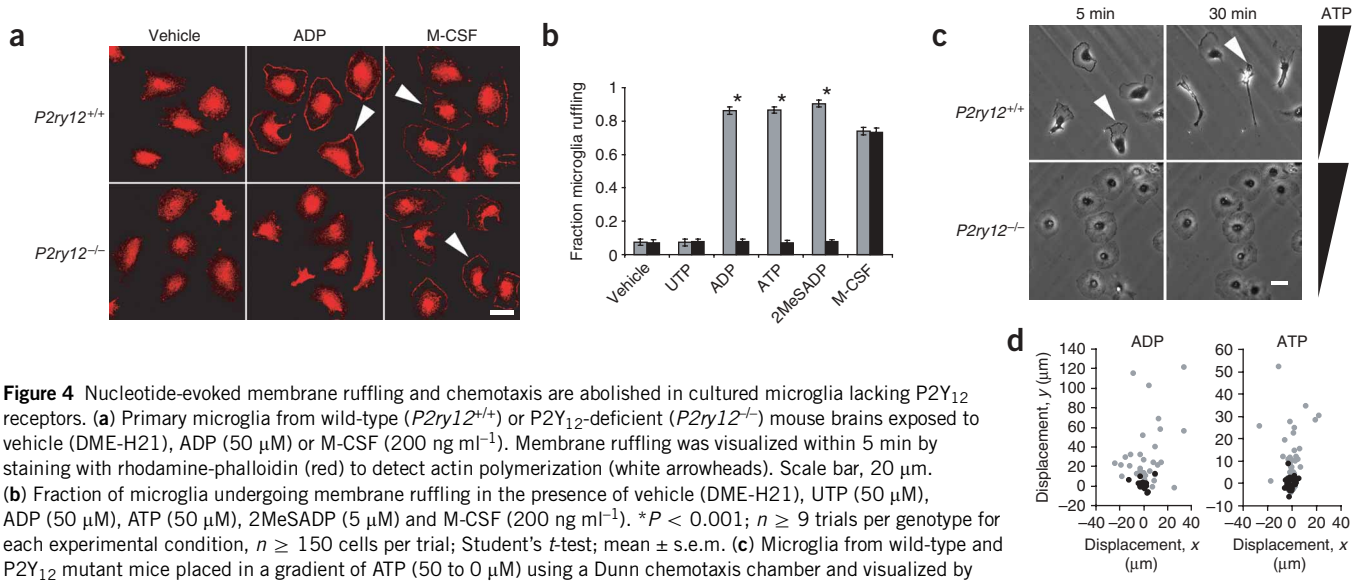

Figure 4 Nucleotide-evoked membrane ruffling and chemotaxis are abolished in cultured microglia lacking $P2Y_{12}$ receptors. (**a**) Primary microglia from wild-type (*P2ry12*$^{+/+}$) or $P2Y_{12}$-deficient (*P2ry12*$^{-/-}$) mouse brains exposed to vehicle (DME-H21), ADP (50 μM) or M-CSF (200 ng ml^{-1}). 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 (DME-H21), UTP (50 μM), ADP (50 μM), ATP (50 μM), 2MeSADP (5 μM) and M-CSF (200 ng ml^{-1}). *$P < 0.001$; $n \geq 9$ trials per genotype for each experimental condition, $n \geq 150$ cells per trial; Student's *t*-test; mean ± s.e.m. (**c**) Microglia from wild-type and $P2Y_{12}$ mutant mice 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 indicating distance (μm) traveled 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* and *y* directions relative to location of the nucleotide source.

$P2Y_{12}$-deficient microglia (~74%), demonstrating that the absence of $P2Y_{12}$ does not generally disrupt signaling mechanisms required for actin polymerization (**Fig. 4a**,**b**). Notably, preincubation with LPS (0.1 mg ml^{-1}) significantly diminished the nucleotide-evoked membrane ruffling of wild-type microglia *in vitro* (**Supplementary Fig. 2** online).

To examine directed motility in response to a localized nucleotide source, we placed purified microglia in a Dunn chemotaxis chamber and observed their behavior in a gradient of ADP or ATP (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. 4c**,**d**; **Supplementary Video 1** online).

We next asked whether similar phenotypes would be observed when microglia were 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 toward the periphery of the slice. In brain slices from neonatal (P4–7) wild-type mice, microglia demonstrated robust process extension and cellular migration toward 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. 5**, **Supplementary Video 2** online). In fact, microglia from mutant mice retracted their processes and traveled toward the injured neuronal cell body layer of the CA3, consistent with the actions of microglia in wild-type slices in the absence of exogenous nucleotides[25].

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[1], microglia in adult wild-type mice 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 period after injection, demonstrating that the $P2Y_{12}$ receptor is absolutely required for ATP-mediated process extension of microglia *in vivo* (**Fig. 6a**,**b** and **Supplementary Video 3** online).

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 showed 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 indicate that $P2Y_{12}$ may be important in this process at all stages of postnatal development on through adulthood.

Morphological response to cortical damage requires $P2Y_{12}$

Tissue damage and loss of cellular integrity can lead to the release of nucleotides and other intracellular factors[26], 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, such as that inflicted by focal laser ablation[1,2]. 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 those from *P2ry12*$^{-/-}$ mice showed dramatically reduced chemotactic responses over an equivalent 40-min period. Interestingly, when microglia from mutant mice were examined 2 h after injury, the degree of process extension into the damaged area approached that observed in wild-type controls at the 32-min time-point (**Fig. 6c**,**d** and **Supplementary Video 4** online), demonstrating that lack of $P2Y_{12}$ receptors significantly delays, but does not abolish, the ability of microglia to respond to local tissue damage.

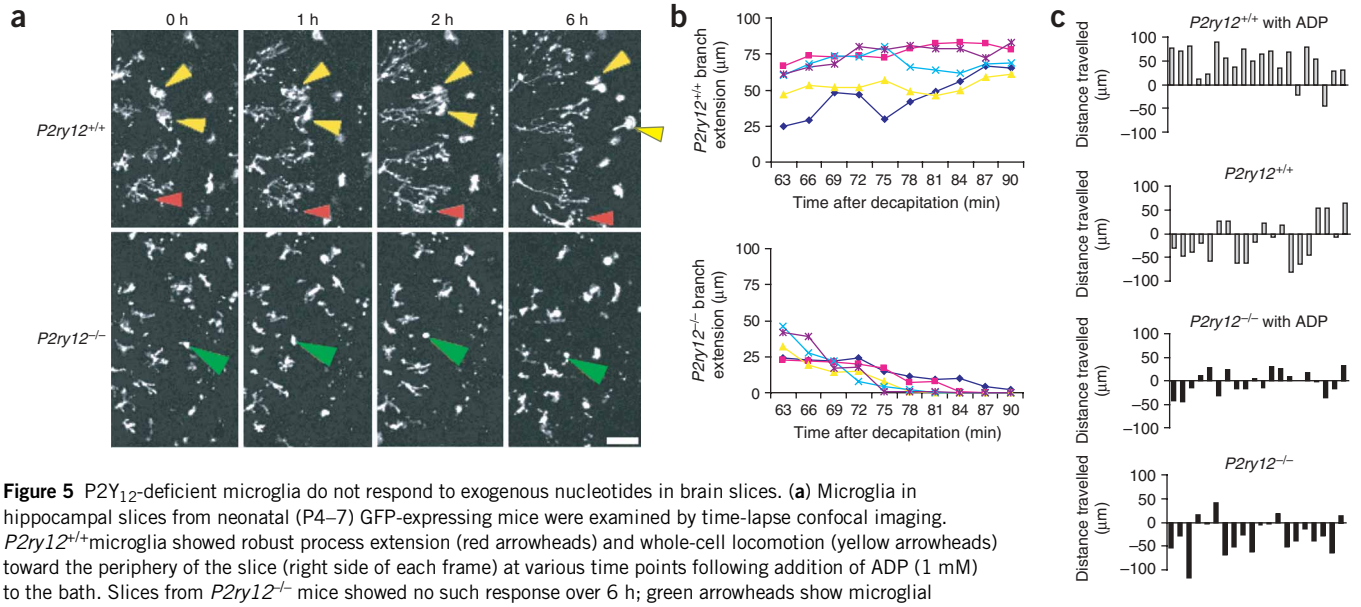

Figure 5 $P2Y_{12}$-deficient microglia do not respond to exogenous nucleotides in brain slices. (**a**) Microglia in hippocampal slices from neonatal (P4–7) GFP-expressing mice were examined by time-lapse confocal imaging. *P2ry12*$^{+/+}$microglia showed robust process extension (red arrowheads) and whole-cell locomotion (yellow arrowheads) toward 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 toward the CA3 pyramidal cell body layer in *P2ry12*$^{-/-}$slice. (**b**) Microglial branch length (μm) in wild-type and $P2Y_{12}$-deficient slices measured at each time-point indicated in the presence of 1 mM ADP ($n = 5$ branches per cell). Positive length change indicates extension, whereas negative length change indicates retraction. Note that all wild-type branches extended, whereas all $P2Y_{12}$-deficient branches retracted 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 traveled (μm) indicates net cell body displacement toward (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 strata pyramidale and oriens of the CA3 area of the hippocampus were examined over a 30-min period.

Recent imaging studies have shown that even in the absence of an injury stimulus, microglia exhibit appreciable, but undirected, process extension, possibly representing homeostatic surveillance of their environment[1,2]. 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[1]. To ask whether this baseline motility requires functional $P2Y_{12}$ receptors, we analyzed the absolute length change of randomly selected processes from wild-type or $P2Y_{12}$-deficient microglia. No significant differences were observed between genotypes (**Supplementary Fig. 1**), suggesting that $P2Y_{12}$ is involved primarily in mediating responses to cellular injury.

DISCUSSION

It has recently been shown that ATP functions as an important signaling 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, in which 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 extracellular nucleotides have an important role in modulating microglial response to CNS damage.

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 by means of 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 signaling[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.

Like platelets, microglia express a plethora of metabotropic and ionotropic purinergic receptor subtypes[6], suggesting that nucleotides have 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[27] and the release of neuroprotective or pro-inflammatory agents, such as tumor necrosis factor-α and interleukin-1β[28,29]. In addition to $P2Y_{12}$, recent studies have identified a second ADP-activated G_i-coupled receptor, $P2Y_{13}$, with similar pharmacological properties[30]. While we could detect low levels of $P2Y_{13}$ transcript in microglia, we were unable to observe protein expression *in vivo* using a $P2Y_{13}$-specific antibody (**Supplementary Fig. 3** online). These results, together with our functional studies, imply that $P2Y_{12}$ is a primary site through which nucleotides mediate the rapid microglial

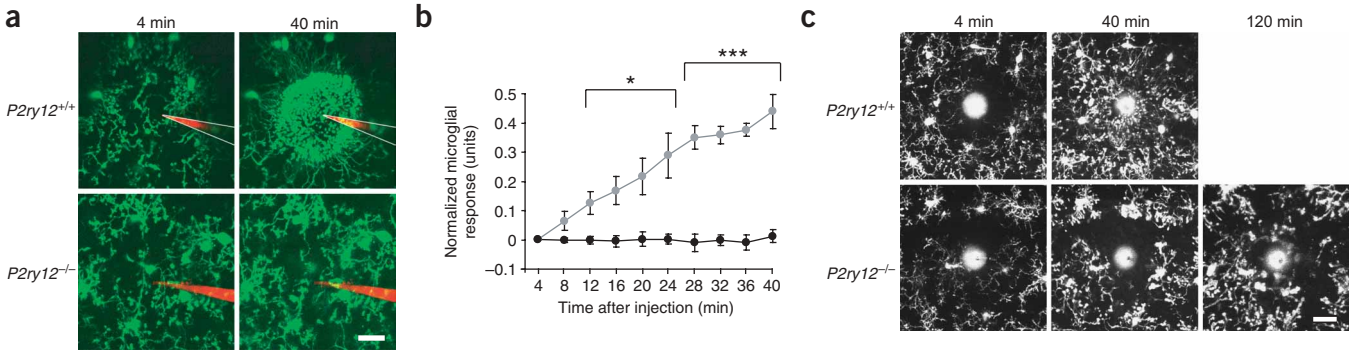

Figure 6 Loss of $P2Y_{12}$ receptors abrogates response of microglia to ATP injection or focal laser ablation in the living brain. (**a**) Microglia from wild-type (*P2ry12*$^{+/+}$) or $P2Y_{12}$-deficient (*P2ry12*$^{-/-}$) GFP-expressing mice visualized *in vivo* using two-photon time-lapse microscopy. Exuberant process extension toward 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 (gray) and $P2Y_{12}$-deficient (black) brains over a 40-min time course ($n = 4$ mice per genotype). (**c**) Tissue ablation by the two-photon laser (white zone in center) results in microglial process extension toward the site of injury. Microglia from mutant mice showed greatly reduced responses over a 40-min observation period. Scale bar, 20 μm. (**d**) Quantification of process extension toward the site of laser ablation ($n = 4$ mice per genotype). $P2Y_{12}$-deficient mice were further analyzed 2 h after injury ($n = 3$). For **b** and **d**, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test; mean ± s.e.m.

responses that we describe here, although a contribution by $P2Y_{13}$ cannot be formally excluded.

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 signaling, apyrase or a saturating concentration of ATP[1]. Thus, it has been proposed that microglial response to injury involves nucleotide signaling through one or more purinergic receptors. Our results support this hypothesis and demonstrate that the $P2Y_{12}$ receptor has an important role in this response. Although $P2Y_{12}$-deficient mice have 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 signaling mechanisms compensate for loss of $P2Y_{12}$ *in vivo*. Other purinergic receptor subtypes may account for such compensation, but signaling by non-nucleotide factors (such as M-CSF, cannabinoids or fractalkine) seems more likely, as $P2Y_{12}$-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 has an important role in regulating initial morphological changes in platelets as they transform from an inactive to active state[12,14,15]. In humans or mice, loss of $P2Y_{12}$ function results in prolonged bleeding times, but platelets can eventually clot because other factors (such as thromboxane A_2) also contribute to the activation process[14]. Despite such functional redundancy, drugs that inhibit platelet $P2Y_{12}$ receptors (such as clopidogrel) have significant cardioprotective effects by decreasing the recurrence of stroke and heart attack, illustrating the importance of this receptor to the overall response to tissue injury.

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 has 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, despite the apparent redundancy in mechanisms leading to microglial activation.

METHODS

Mouse strains. *P2ry12*$^{-/-}$ mice[14] were mated with *Cx3cr1*$^{+/GFP}$ animals[18] to generate paired *P2ry12*$^{+/+}$*Cx3cr1*$^{+/GFP}$ and *P2ry12*$^{-/-}$*Cx3cr1*$^{+/GFP}$ littermates for these studies. Mice were housed and bred at the University of California, San Francisco animal facilities, and all experiments were performed in accordance with protocols approved by Animal Use Committees of UCSF, Skirball Institute, and the University of Iowa.

Immunohistochemistry. Polyclonal antibody to $P2Y_{12}$ receptor (anti-$P2Y_{12}$) 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). 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-month-old) mice was fixed with 4% paraformaldehyde and sectioned (30 μm thick) on a cryotome, blocked for 30 min in 10% normal goat serum, 0.1% triton in PBS and stained with primary antibodies to $P2Y_{12}$, CD11b (eBiosciences) or GFAP (Pharmingen) overnight at 4 °C, washed and visualized with Alexa 546– or Alexa 594–conjugated goat antibody to rabbit IgG (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 a ×40 oil- or ×20 multi-immersion objective, or a Nikon wide-field fluorescence microscope using a ×20 lens.

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[31]. Briefly, brains were

dissected, homogenized by passing through an 18 G needle and cultured in Dulbecco's modified Eagle's medium with high glucose (DME-H21) with 10% heat-inactivated FBS and penicillin plus 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 onto 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[10] by replacing DME-H21 with fresh DME-H21 containing no stimulus (negative control) or ADP, ATP or UTP (50 μM), 2MeSADP (0.5 μM, Sigma) or M-CSF (200 ng ml^{-1}, 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). Chemotaxis assays were performed using the Dunn chamber[32] 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 (from 0 to 0.8 μm) was subtracted to obtain final values. Images were processed and analyzed using the US National Institutes of Health (NIH) ImageJ software.

***Ex vivo* hippocampal slice preparation and imaging.** Hippocampal tissue slices (400 μm thick) were prepared from P4–7 mice and stained as described previously[25,33] 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 were imaged on a Zeiss LSM 510 Meta, Leica TCS NT or Leica SP2 AOBS confocal microscope with a ×20 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).

To examine the microglial response to applied nucleotides, acutely prepared slices were 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[25]. 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 ×20 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[1,2]. 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[1]. Changes in GFP fluorescence were monitored as processes entered a circular zone 70 μm in diameter centered over the injection needle and normalized to an outer region 136 μm in diameter. Microglial response to laser ablation was quantified similarly using a circular zone 35 μm in diameter centered on the ablation and normalized to an outer region 97 μm in diameter.

LPS injections and analysis of $P2Y_{12}$ expression. Mice were anesthetized with ketamine and xylazine 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^{-1} LPS (Sigma) or PBS (control) was bilaterally injected into the striatum. The wound was closed and mice allowed to recover for 4 d before 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 digoxigenin-labeled cRNA probe containing the entire mouse $P2Y_{12}$ coding region (*Sac*I–*Nsi*I 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[34] using a ^{32}P-labeled 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′-GGCTCCCAG TTTAGCATCAC-3′. Quantification of amplified products was carried out as previously described[34].

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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