

# Adenylate cyclase 5 coordinates the action of ADP, P2Y1, P2Y13 and ATP-gated P2X7 receptors on axonal elongation

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## Summary

In adult brains, ionotropic or metabotropic purinergic receptors are widely expressed in neurons and glial cells. They play an essential role in inflammation and neurotransmission in response to purines secreted to the extracellular medium. Recent studies have demonstrated a role for purinergic receptors in proliferation and differentiation of neural stem cells although little is known about their role in regulating the initial neuronal development and axon elongation. The objective of our study was to investigate the role of some different types of purinergic receptors, P2Y1, P2Y13 and P2X7, which are activated by ADP or ATP. To study the role and crosstalk of P2Y1, P2Y13 and P2X7 purinergic receptors in axonal elongation, we treated neurons with specific agonists and antagonists, and we nucleofected neurons with expression or shRNA plasmids. ADP and P2Y1–GFP expression improved axonal elongation; conversely, P2Y13 and ATP-gated P2X7 receptors halted axonal elongation. Signaling through each of these receptor types was coordinated by adenylate cyclase 5. In neurons nucleofected with a cAMP FRET biosensor (ICUE3), addition of ADP or Blue Brilliant G, a P2X7 antagonist, increased cAMP levels in the distal region of the axon. Adenylate cyclase 5 inhibition or suppression impaired these cAMP increments. In conclusion, our results demonstrate a crosstalk between two metabotropic and one ionotropic purinergic receptor that regulates cAMP levels through adenylate cyclase 5 and modulates axonal elongation triggered by neurotrophic factors and the PI3K–Akt–GSK3 pathway.

**Key words:** Axon elongation, Purinergic receptor, Adenylate cyclase

## Introduction

Most early studies of the roles of nucleotides in development have examined their intracellular roles. However, it is now generally accepted that purines and pyrimidines have potent extracellular actions mediated by the activation of specific membrane receptors. The role of purinergic signaling during developmental and pathological states is only now beginning to be explored because of the large number of purinergic receptor subtypes involved and because purines have effects on every major cell type present in the CNS. ATP is released by different cell types in response to multiple physiological stimuli, as well as after programmed or injury-induced cell death, and can be converted into other purines, such as ADP, AMP or adenosine by the action of extracellular ectonucleotidases (Burnstock, 2007). ATP, ADP or adenosine can activate purinergic receptors, which are subdivided in P1 and P2 receptors. The P2 receptor family consists of cationic ATP-operated P2X receptors, and the metabotropic G-protein-coupled P2Y receptors, which are activated by different purines and pyrimidines, among them ADP (Abbracchio et al., 2009).

Although our understanding of the pathways of intercellular purinergic signaling is still limited, it is clear that purinergic signaling represents a main non-synaptic signaling mechanism in the normal and diseased CNS. Acting at purinergic receptors, extracellular purines can regulate neurotransmission and developmental events, such as cell migration or apoptosis, and they have been implicated in several central nervous system disorders (Burnstock, 2007; Burnstock, 2008). Recent studies have shown that inhibition of the ATP-gated P2X7 receptor improves recovery after spinal cord injury (Peng et al., 2009; Wang et al., 2004), promotes axonal growth in hippocampal neurons (Diaz-Hernandez et al., 2008) and induces N2a cell differentiation (Gomez-Villafuertes et al., 2009).

The regulation of axonal elongation is an important feature during neuronal development and axonal recovery after axonal injury, in order to achieve functional neuronal connectivity. Neurotrophic factors, axon guidance molecules and neurotransmitter receptors play an essential role in this process (Huang and Reichardt, 2001; Mueller, 1999; Ruediger and Bolz,

2007). However, the potential role in nervous system development of purinergic receptors and more precisely that of dinucleoside polyphosphates, such as ADP, which can activate various P2Y receptors, has not yet been investigated. The coordination of purinergic receptors specific for different purines also requires further investigation.

The P2Y receptor family can be subdivided into two subgroups: Gq- or G11-coupled P2Y1, 2, 4, 6 and 11 receptors; and Gi- or Go-coupled P2Y12, 13 and 14 receptors (Abbraccio et al., 2006; Burnstock, 2007). P2Y receptors are expressed in the CNS, in both glia and neurons and they are known to couple to neuronal ion channels (Boehm, 2003) and to modulate pain responses (Malin and Molliver, 2010). P2Y1, P2Y12 and P2Y13 are preferentially activated by ADP (Burnstock, 2007). The P2Y1 receptor plays an important role in neurosphere proliferation (Mishra et al., 2006) and the radial migration of cortical neurons (Liu et al., 2008). P2Y13 is also implicated in regulating CNS neurons (Ortega et al., 2008). Both P2Y1 and P2Y13 are expressed in hippocampal neurons (Csolle et al., 2008) and the Allen Brain Atlas; <http://www.brain-map.org/>), whereas P2Y12 has been described in rat brainstem, dorsal root ganglion neurons (Heinrich et al., 2008) and oligodendrocytes, but it is absent from hippocampal and neocortical neurons (Amadio et al., 2006; Hollopeter et al., 2001).

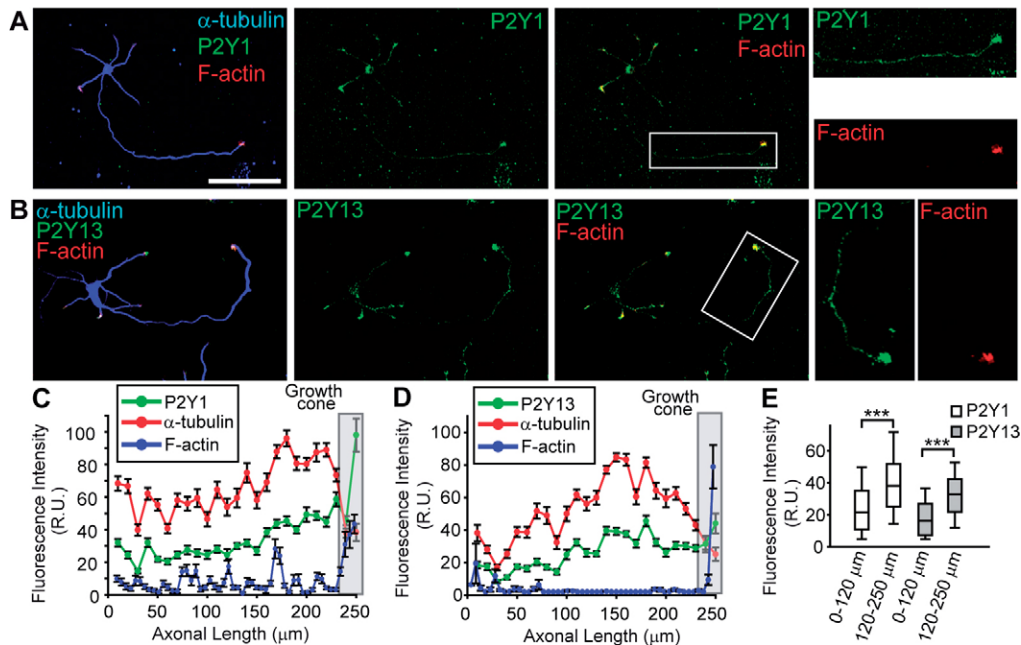
In the context of axonal growth improvement after axonal injury, we have employed a widely used model of embryonic cultured hippocampal neurons with the objective of understanding how different purines and purinergic receptors, such as P2X7, P2Y1 or P2Y13, can regulate axonal elongation and how they are coordinated. Because these receptors are widely expressed in neurons and glial cells, the study was performed in a

widely used model of pure cultured hippocampal neurons in the absence of glial cells. We show that ADP promotes axonal elongation through the P2Y1 receptor, whereas the P2Y13 receptor exerts a negative effect on axonal elongation, as described previously for the ATP-gated P2X7 receptor (Diaz-Hernandez et al., 2008). All three receptors are expressed in the distal region of the axon and modulate signaling pathways that involve extracellular calcium influx (assisted by P2X7) and the Gq (P2Y1) and Gi (P2Y13) proteins. The coordinated action of these three signaling pathways contributes to the fine regulation of adenylyl cyclase 5 (AC5, also known as ADCY5), which modulates cAMP levels and axon elongation by modulating, the PI3K–Akt–GSK3 pathway.

## Results

### ADP regulates axonal elongation in cultured hippocampal neurons through the P2Y1 and P2Y13 receptors

In the light of previous results demonstrating how ATP modulates axon elongation through the ionotropic P2X7 receptor (Diaz-Hernandez et al., 2008), we analyzed whether ADP, acting through the metabotropic P2Y1 and P2Y13 receptors, can regulate axonal elongation. Both these receptors are expressed in the hippocampus and hippocampal neurons (supplementary material Fig. S1) and are specific for ADP (Burnstock, 2007). In our model of cultured hippocampal neurons, both receptors were found in the axon, with a more intense signal in the distal region of the axon (Fig. 1). The fluorescence of both P2Y1 and P2Y13 colocalized with microtubules in the distal region of the axon and with the actin region of growth cones. Both receptors were also detected in the neuronal soma and to a lesser extent in the future dendrites.



**Fig. 1. P2Y1 and P2Y13 receptors are localized in the distal axon of cultured hippocampal neurons.** (A,B) Hippocampal neurons after 3 DIV, stained with antibodies against  $\alpha$ -tubulin (blue) and Alexa-Fluor-594–phalloidin (red) to visualize neuronal morphology, and P2Y1 (A) or P2Y13 (B, green). Scale bar: 100  $\mu$ m. Boxes indicate the area of the distal region of the axon magnified in the panels on the right. (C,D) Graphs of P2Y1 and P2Y13 fluorescence (means  $\pm$  s.e.m.) in 10  $\mu$ m sections along the length of the axon. Axons approximately 250  $\mu$ m long were analyzed in five neurons in two separate experiments. (E) Box-plot showing the distribution of fluorescence intensities of P2Y1 and P2Y13 receptors in the proximal (0–120  $\mu$ m) and distal (120–250  $\mu$ m) regions of the axon. There was a significant increased in the fluorescence signal for both receptors in the distal region of the axon compared with the proximal region; \*\*\* $P$ <0.001.

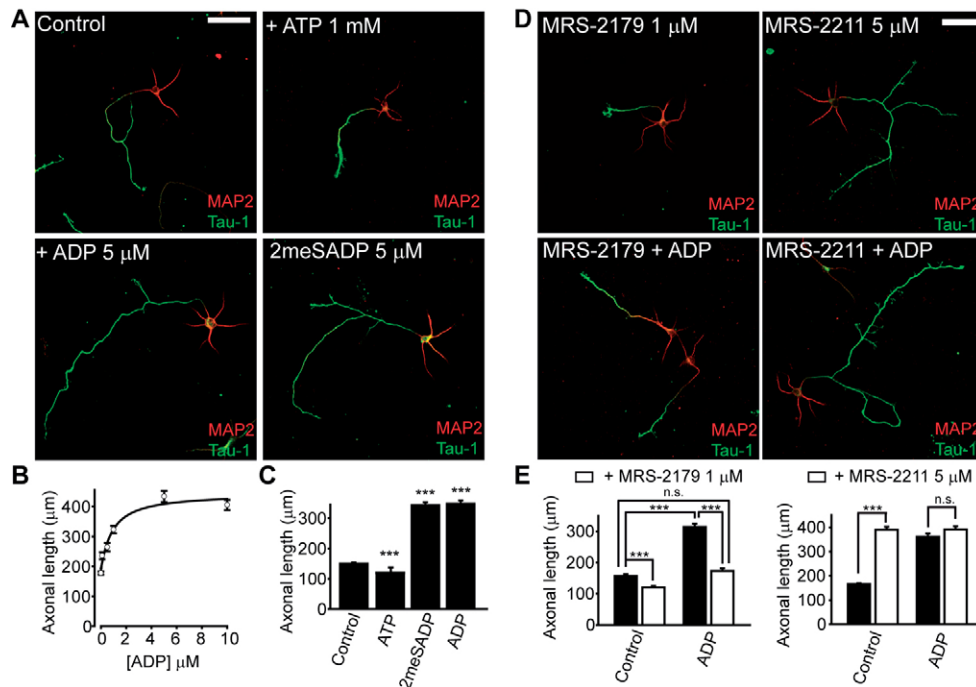
The influence of ADP on axon elongation was examined in hippocampal neurons treated with increasing concentrations of ADP (1 nM, 100 nM, 500 nM, 1  $\mu$ M, 5  $\mu$ M or 10  $\mu$ M) on the first day in vitro (1 DIV) until 3 DIV (Fig. 2B). ADP significantly increased axon length when compared with control untreated neurons ( $177.88 \pm 5.6 \mu\text{m}$ ), with a maximum effect at 5  $\mu$ M ( $433.55 \pm 18.69 \mu\text{m}$ ) and an  $\text{ED}_{50}$  of  $\sim 1 \mu\text{M}$  (Fig. 2A,B). We then assessed the effect of ADP (5  $\mu$ M) or 2-MeSADP (5  $\mu$ M) on axon length, the latter is an ADP analogue with equal or greater specificity for P2Y1 and P2Y13 purinergic receptors (Fig. 2A,C). Both 2-MeSADP and ADP enhanced axon growth to a similar extent ( $342.76 \pm 9.99 \mu\text{m}$  and  $347.70 \pm 11.12 \mu\text{m}$ , respectively) compared with control neurons ( $150.47 \pm 3.42 \mu\text{m}$ ). By contrast, the addition of 1 mM ATP to cultured hippocampal neurons in the same conditions retarded axonal growth ( $114.12 \pm 4.97 \mu\text{m}$  vs  $174.25 \pm 6.98 \mu\text{m}$  in control neurons; Fig. 2A,C).

#### Activation of P2Y1 and P2Y13 receptors by ADP exerts opposing effects on axon elongation

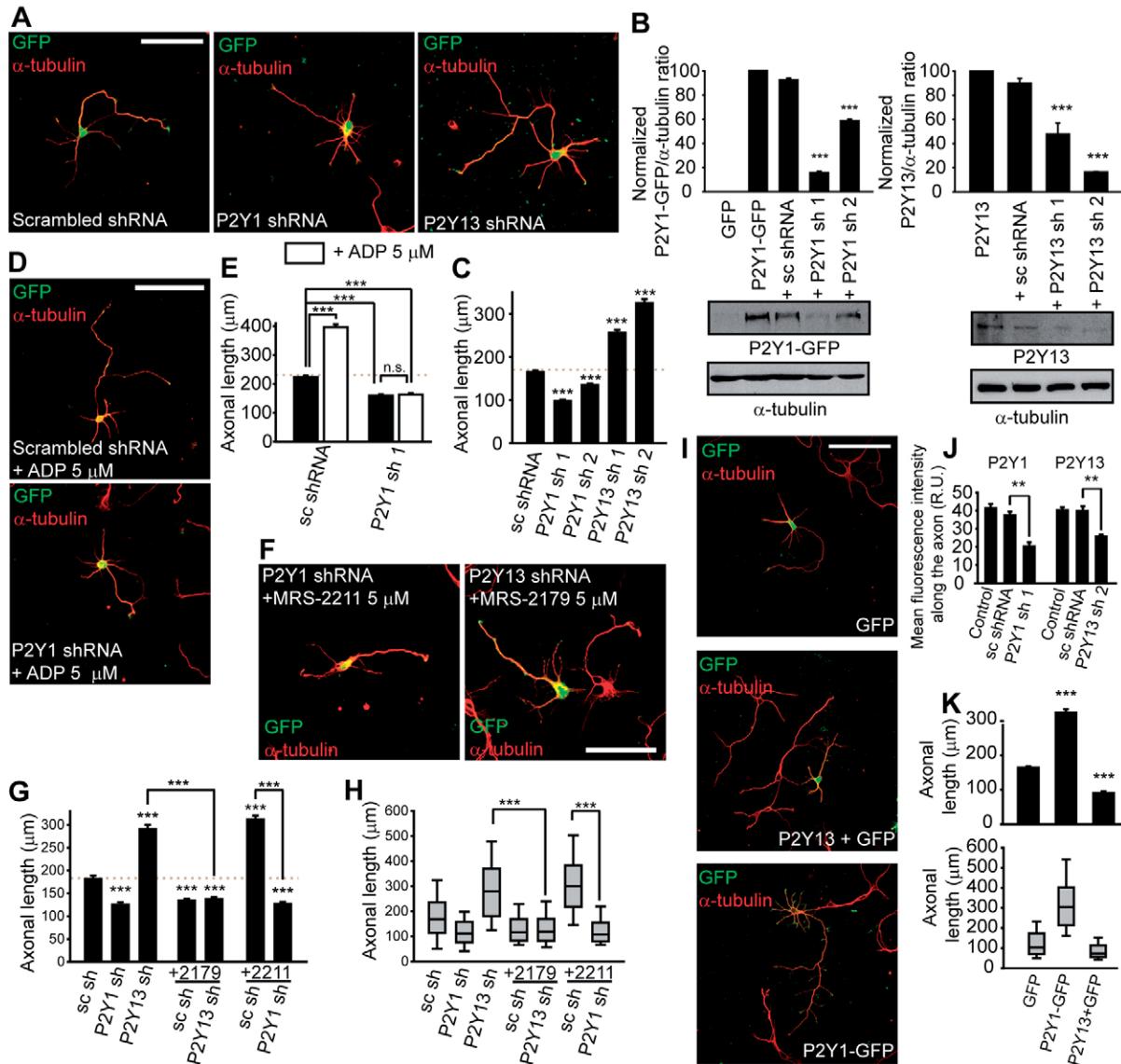
To identify the role of each receptor in axon elongation neurons were cultured for 3 DIV and treated for the last 48 hours with the P2Y1 antagonist, MRS-2179 or the P2Y13 antagonist, MRS-2211 at different concentrations (supplementary material Fig. S2). Treatment with the P2Y1 antagonist MRS-2179 significantly retarded axonal growth ( $86.62 \pm 2.30 \mu\text{m}$  at 0.5  $\mu\text{M}$  and

$70.54 \pm 1.94 \mu\text{m}$  at 1  $\mu\text{M}$ , respectively), and treatment with the P2Y13 antagonist MRS-2211 (1, 5 or 10  $\mu\text{M}$ ) significantly increased axonal growth ( $379.48 \pm 12.99 \mu\text{m}$ ,  $405.39 \pm 13.14 \mu\text{m}$  and  $428.39 \pm 13.68 \mu\text{m}$ , respectively) compared with that of control neurons ( $150.47 \pm 3.42 \mu\text{m}$ ), suggesting that the P2Y13 receptor negatively regulates axon elongation. Moreover, neurons cultured in the presence of MRS-2179 (1  $\mu\text{M}$ ) and ADP (5  $\mu\text{M}$ ) did not generate significantly longer axons ( $173.14 \pm 8.47 \mu\text{m}$ ) than control neurons ( $156.92 \pm 5.69 \mu\text{m}$ ), indicating that the influence of ADP on axon elongation is mediated by the P2Y1 receptor (Fig. 2D,E). Furthermore, the increase in axon length in the presence of both ADP and MRS-2211 was similar to that when either compound was used alone (Fig. 2D,E).

The effects of the specific antagonists of P2Y1 and P2Y13 were confirmed by interference short hairpin RNAs (shRNAs) for P2Y1 or P2Y13 receptors (Fig. 3). The axons produced by neurons nucleofected with the scrambled (control) shRNA plasmid reached a length of  $164.53 \pm 3.73 \mu\text{m}$ , whereas nucleofection with P2Y1 shRNA 1 or P2Y1 shRNA 2 resulted in lengths of  $97.60 \pm 3.25 \mu\text{m}$  and  $134.66 \pm 3.78 \mu\text{m}$ , respectively (Fig. 3A,C). This reduction in axonal growth was correlated with a decrease in P2Y1 protein expression produced by each shRNA (Fig. 3B,J). Moreover, treatment with ADP (5  $\mu\text{M}$ ) promoted axonal growth in neurons nucleofected with the scrambled shRNA ( $395.67 \pm 10.57 \mu\text{m}$ ) but not in those nucleofected with P2Y1 shRNA 1 ( $163.29 \pm 5.22 \mu\text{m}$  vs  $223.53 \pm 5.37 \mu\text{m}$  in scrambled-shRNA-nucleofected neurons:



**Fig. 2. ADP modulates axon elongation through the P2Y1 and P2Y13 purinergic receptors.** (A) Hippocampal neurons cultured in the presence or absence of ADP (5  $\mu\text{M}$ ), ATP (1 mM) or the ADP analogue 2MeSADP (5  $\mu\text{M}$ ), from day 1 to day 3 in vitro. Neurons were fixed at 3 DIV and stained with antibodies against MAP2 (red, somatodendritic) and Tau-1 (green, axon) to define the neuronal morphology and quantify axon length. (B) Axon length of hippocampal neurons at 3 DIV following treatment with increasing concentrations of ADP (0.1, 0.5, 1, 5 and 10  $\mu\text{M}$ ). The resulting curve revealed an  $\text{ED}_{50}$  of 0.879  $\mu\text{M}$ , with a maximum effect at 5  $\mu\text{M}$  ADP. (C) Axon length at 3 DIV following treatment with ADP (5  $\mu\text{M}$ ), 2MeSADP (5  $\mu\text{M}$ ) and ATP (1 mM). Data are means  $\pm$  s.e.m. from three independent experiments from 100 neurons analyzed in each experiment and condition. (D) Hippocampal neurons incubated with the P2Y1 antagonist (MRS-2179) or the P2Y13 antagonist (MRS-2211) from day 1 to day 3 in vitro, in the presence or absence of ADP (5  $\mu\text{M}$ ). Neurons were stained with MAP2 and Tau-1 antibodies, and the length of their axon was quantified. (E) Graphs of the mean axon lengths  $\pm$  s.e.m. of the control and treated neurons shown in D. All data are the means  $\pm$  s.e.m. from three independent experiments with 100 neurons analyzed in each experiment and condition; \*\*\* $P < 0.001$ . n.s., non-significant differences. Scale bars: 50  $\mu\text{m}$ .



**Fig. 3. P2Y1 silencing impairs ADP-dependent axon elongation.** (A) Hippocampal neurons were nucleofected with scrambled shRNA, P2Y1 shRNA or P2Y13 shRNA. Neurons were fixed at 3 DIV and stained with an anti- $\alpha$ -tubulin antibody. Nucleofected neurons were identified by their GFP fluorescence. (B) HEK-293T cells were co-transfected with GFP, P2Y1-GFP or P2Y13 plasmids, in combination with different P2Y1 or P2Y13 shRNAs. Data are means  $\pm$  s.e.m. of three independent experiments. P2Y1-GFP and P2Y13 protein expression was normalized to  $\alpha$ -tubulin expression levels; \*\*\* $P$ <0.001. (C) Axon length of hippocampal neurons expressing scrambled shRNA, two different P2Y1 shRNAs or two different P2Y13 shRNAs was quantified after staining with antibodies against MAP2 and Tau-1. Data are mean axon lengths  $\pm$  s.e.m. from three independent experiments, analyzing 100 neurons for each condition in each experiment; \*\*\* $P$ <0.001. The dotted grey line indicates the mean axon length of scrambled-shRNA-nucleofected neurons. (D,E) Hippocampal neurons nucleofected with scrambled shRNA or P2Y1 shRNA and treated with ADP (5  $\mu$ M) from day 1 to day 3 in vitro. The graph in D shows the axon length in nucleofected neurons (GFP-positive) incubated in the presence or absence of ADP. (F-H) Hippocampal neurons nucleofected with scrambled shRNA, P2Y1 shRNA or P2Y13 shRNA and treated with the P2Y1 antagonist (MRS-2179) or the P2Y13 antagonist (MRS-2211) from day 1 to day 3 in vitro. Scale bars: 50  $\mu$ m. Note that in all cases P2Y1 expression and function is necessary for axon elongation. Data in G are the mean axon lengths  $\pm$  s.e.m. from three independent experiments, analyzing 100 neurons for each condition in each experiment; \*\*\* $P$ <0.001. H shows the distribution of the axon length for all neurons from three independent experiments for each condition ( $n$ =300). (I) Hippocampal neurons that had been nucleofected with plasmids expressing GFP, P2Y1-GFP and P2Y13. After 3 DIV neurons were stained for MAP2 and Tau-1 to identify the axon. (J) P2Y1 or P2Y13 mean fluorescence intensity along the axon in control, scrambled shRNA, P2Y1 shRNA or P2Y13 shRNA nucleofected neurons. (K) Graph of the mean axon lengths  $\pm$  s.e.m. of neurons nucleofected with GFP, P2Y1-GFP or P2Y13 and GFP. Neurons were quantified in three independent experiments, analyzing 100 neurons for each condition in each experiment; \*\*\* $P$ <0.001. Scale bars: 100  $\mu$ m. Box-plot shows the distribution of axon lengths for all the neurons quantified in K.

Fig. 3D,E). In contrast to the P2Y1 shRNAs, nucleofection with plasmids expressing P2Y13 shRNA 1 or P2Y13 shRNA 2 (Fig. 3A,C) approximately doubled the length of axons ( $255.79 \pm 7.17 \mu\text{m}$  or  $325.05 \pm 9.23 \mu\text{m}$ , respectively) when

compared with neurons nucleofected with scrambled shRNA ( $164.53 \pm 3.73 \mu\text{m}$ ).

In accordance with these results, the expression of P2Y1-GFP increased mean axon length to  $324.24 \pm 9.24 \mu\text{m}$  (Fig. 3I,K),



whereas co-nucleofection with plasmids expressing P2Y13 and GFP resulted in a mean axon length of  $91.36 \pm 4.76 \mu\text{m}$ ; the axon length of the control neurons was  $164.53 \pm 3.73 \mu\text{m}$ .

We analyzed the relative contributions of P2Y1 activation and P2Y13 inhibition to axon growth (Fig. 3F–H) by nucleofecting neurons with the scrambled shRNA or P2Y13 shRNA 2 and treating them with the P2Y1 antagonist, MRS-2179. The increase in axon length following P2Y13 suppression ( $290.43 \pm 9.86 \mu\text{m}$  vs  $181.70 \pm 6.96 \mu\text{m}$  in non-treated scrambled shRNA nucleofected neurons) was blocked by P2Y1 antagonism ( $137.08 \pm 4.67 \mu\text{m}$ ). Similarly, the increase in axonal length seen in neurons treated with the P2Y13 antagonist, MRS-2211, was impaired in neurons expressing P2Y1 interference RNA 1 ( $127.27 \pm 4.14 \mu\text{m}$ ). Taken together, these findings indicate that P2Y1 activation is necessary and sufficient to promote axon elongation, whereas P2Y13 activation might inhibit this effect.

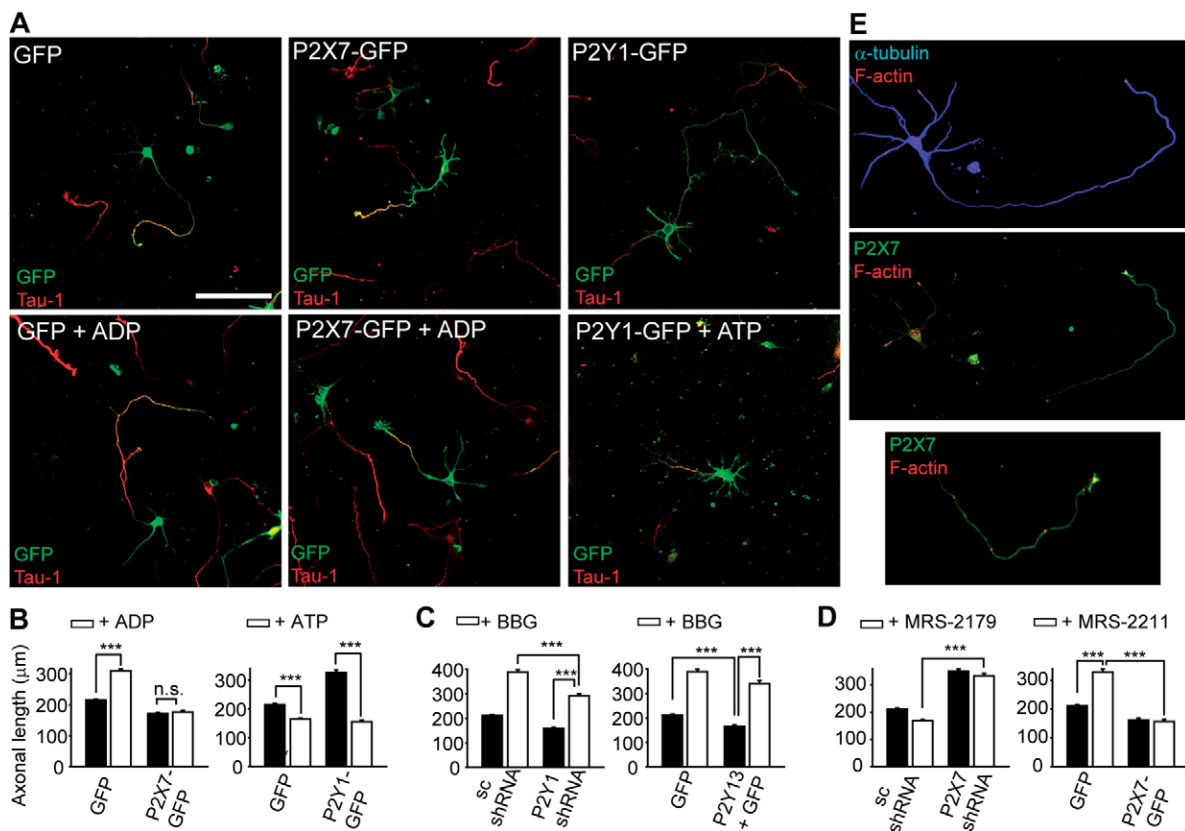
#### ADP-mediated axon elongation is dependent upon the status of P2X7

ATP negatively regulates axon growth through the activation of the P2X7 receptor, whereby the inhibition or suppression P2X7 enhances axon growth. The immunofluorescence pattern of P2X7 revealed the same localization that P2Y1 and P2Y13 receptors in the hippocampus as previously described (Fig. 4E) (Diaz-Hernandez et al., 2008). To understand the relationship between

P2X and P2Y purinergic receptors, hippocampal neurons were nucleofected with a construct expressing the P2X7 receptor coupled to GFP, or with GFP alone, and treated with ADP ( $5 \mu\text{M}$ ) from day 1 to day 3 in vitro, as described above. Interestingly, ADP treatment increased axon length in GFP-nucleofected neurons, but failed to do so in those expressing P2X7–GFP (Fig. 4A,B). In the same sense, axons of P2Y1–GFP-expressing neurons treated with ATP ( $1 \text{ mM}$ ) were shorter than those of untreated GFP-nucleofected neurons (Fig. 4A,B). P2X7 inhibition with Blue Brilliant G (BBG;  $100 \text{ nM}$ ) reversed the slow axon growth seen in neurons expressing either P2Y1 shRNAs or the P2Y13 receptor, resulting in an increase in axon length (Fig. 4C), which was significantly lower than in scrambled shRNA- or GFP-nucleofected neurons treated with BBG. Furthermore, P2X7 shRNAs abolished the effects of inhibiting the P2Y1 receptor with MRS-2179, whereas P2X7–GFP expression impaired the axon growth-promoting effect of the P2Y13 antagonist, MRS-2211 (Fig. 4D). These results suggest that P2X7 activation by ATP blocks an ADP-mediated signaling mechanism that is regulated by P2Y1 and by P2Y13.

#### Adenylate cyclase activity is necessary for the regulation of axonal elongation by P2Y1, P2Y13 and P2X7

To test the hypothesis that a common signaling pathway integrates P2Y1-, P2Y13- and P2X7-mediated signaling, we

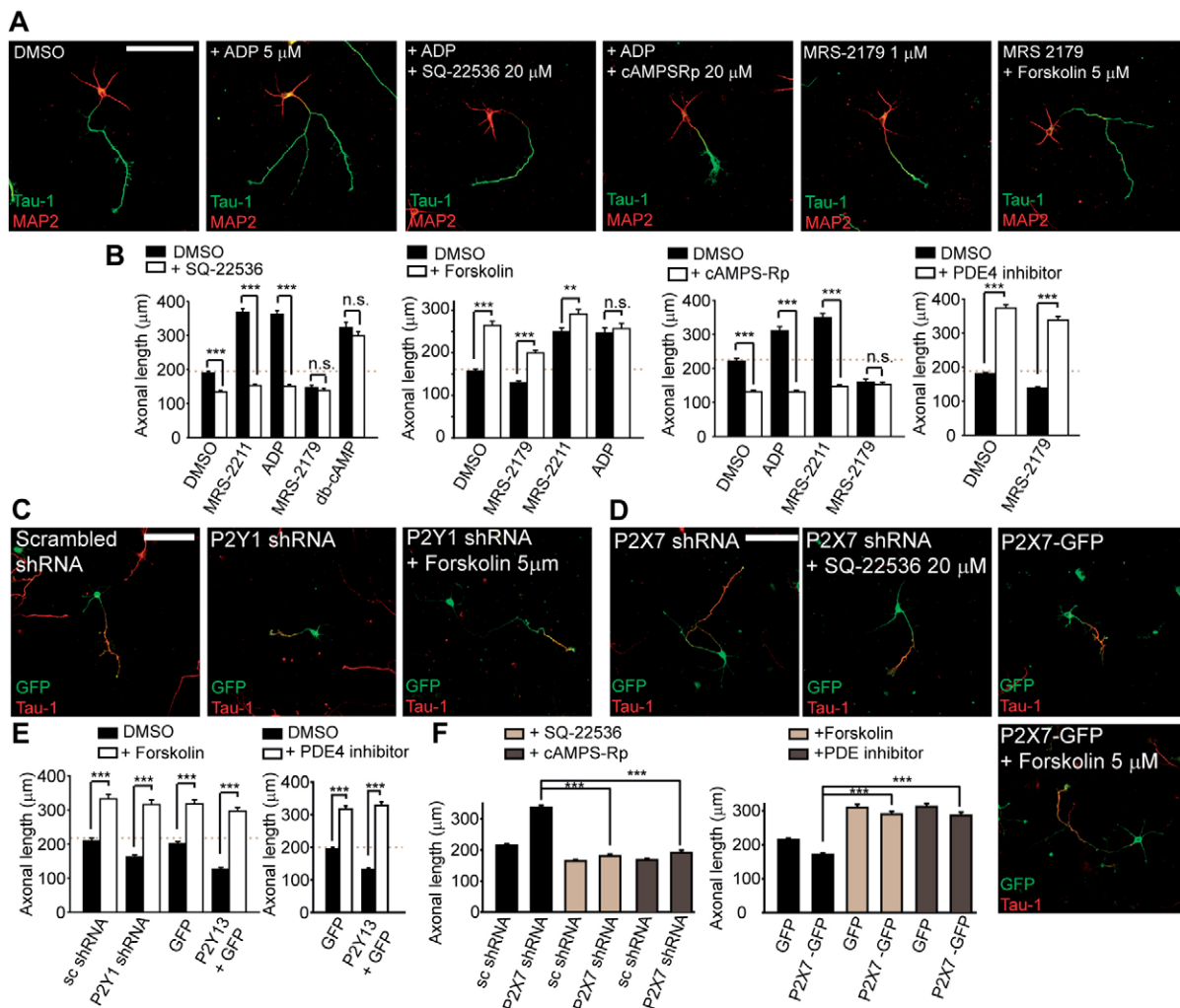


**Fig. 4. P2X7 impairs ADP-P2Y1-dependent axon elongation.** (A) Hippocampal neurons nucleofected with GFP, P2Y1–GFP and P2X7–GFP and cultured in the presence or absence of ADP ( $5 \mu\text{M}$ ) from day 1 to day 3 in vitro. Neurons were stained with an anti-Tau-1 antibody (red) to visualize the axon, and nucleofected neurons were identified by GFP fluorescence. Scale bar:  $100 \mu\text{m}$ . (B–D) Axon length was quantified in neurons nucleofected with the plasmids indicated in each graph. Bars show the mean axon length in neurons treated with (white) or without (black) the indicated P2 agonist or antagonist. Data are the mean axon lengths  $\pm$  s.e.m. from three independent experiments, analyzing 100 neurons for each condition in each experiment; \*\*\* $P < 0.001$ , n.s., not significant. (E) P2X7 expression in a 3 DIV hippocampal neuron stained with anti- $\alpha$ -tubulin (blue), P2X7 (green) and Alexa-Fluor-594-phalloidin (red).

first investigated whether the effects of P2Y1 inhibition or exogenous P2Y13 expression could be reversed by activating or inhibiting their associated G proteins (supplementary material Fig. S3A–D). Treating neurons from day 1 to day 3 in vitro with rPMT (20 ng/ml), a recombinant peptide that activates G<sub>q</sub>, reversed the inhibitory effect of MRS-2179 on P2Y1 and promoted axon growth ( $230.46 \pm 8.54 \mu\text{m}$  vs  $128.69 \pm 4.67 \mu\text{m}$  in MRS-2179-treated neurons or vs  $156.55 \pm 5.25 \mu\text{m}$  in control neurons). Moreover, inhibition of phospholipase C activity, which is activated by G<sub>q</sub>, attenuated axon elongation in the presence of ADP (supplementary material Fig. S3E,F). Inhibition of G<sub>i</sub> with pertussis toxin (PTX) reversed the reduced growth provoked by exogenous P2Y13 expression ( $323.76 \pm 12.22 \mu\text{m}$  vs

$133.67 \pm 6.08 \mu\text{m}$  in exogenous P2Y13-expressing neurons vs  $197.19 \pm 9.24 \mu\text{m}$  in GFP-expressing neurons; supplementary material Fig. S3B,D). On the basis of these findings, we next sought to identify common signaling components that can be regulated in opposite directions by G<sub>i</sub> and G<sub>q</sub> proteins, such as adenylate cyclases 1, 3, 5, 6, 8 and 9 (Willoughby and Cooper, 2007).

We investigated the common signaling hypothesis in hippocampal primary cultures using four different experimental approaches (Fig. 5): (1) administration of an adenylate cyclase inhibitor (SQ-22536;  $20 \mu\text{M}$ ); (2) activation of adenylate cyclase with forskolin ( $5 \mu\text{M}$ ); (3) administration of a competitive antagonist of cAMP-induced activation of PKA (cAMPS-Rp;



**Fig. 5. Adenylate cyclase activity is necessary for ADP–P2Y1-dependent axon elongation.** (A) Hippocampal neurons treated with the indicated compounds from day 1 to day 3 in vitro and stained for MAP2 and Tau-1. Scale bar: 100  $\mu\text{m}$ . (B) Axon length in neurons treated with vehicle (black bars) or the indicated adenylate cyclase or cAMP regulators (white bars), in combination with agonists or antagonists of P2Y1 or P2Y13. Graphs represent the mean axon length  $\pm$  s.e.m. from three independent experiments, analyzing 100 neurons for each condition in each experiment; \*\*\* $P < 0.001$ , \*\* $P < 0.01$ ; n.s., not significant. (C,E) Hippocampal neurons nucleofected with P2Y1 shRNA and stained at 3 DIV for Tau-1 or  $\alpha$ -tubulin (red). Nucleofected neurons were identified by GFP fluorescence. Neurons were treated with the adenylate cyclase activator forskolin ( $5 \mu\text{M}$ ) or a PDE4 inhibitor ( $20 \text{ nM}$ ). Note that both treatments reversed the negative effects of P2Y1 silencing or P2Y13 expression on axon elongation. The graphs in E show the axonal lengths  $\pm$  s.e.m. from three independent experiments, analyzing 100 GFP positive neurons for each condition in each experiment; \*\*\* $P < 0.001$ . (D,F) Neurons nucleofected with P2X7 shRNA or P2X7–GFP expression plasmids and treated from day 1 to day 3 in vitro with the adenylate cyclase inhibitor (SQ-22536) or the adenylate cyclase activator forskolin, respectively. Note that adenylate cyclase activation or increased cAMP levels reversed the negative effect of P2X7–GFP expression on axon elongation. The graphs in F show the axon length  $\pm$  s.e.m. from three independent experiments analyzing 100 neurons for each condition in each experiment; \*\*\* $P < 0.001$ . Scale bars: 100  $\mu\text{m}$ .

20  $\mu\text{M}$ ) or a PKA inhibitor (H-89; 5  $\mu\text{M}$ ); and (4) selective inhibition of the predominant phosphodiesterases in brain, PDE4B and PDE4D (Iona et al., 1998) with 3,5-dimethyl-1-(3-nitrophenyl)-1H-pyrazole-4-carboxylic acid ethyl ester (20 nM). These treatments were administered in combination with different agonists or antagonists of P2Y1 and P2Y13 receptors (Fig. 5A,B), as well as in cells in which P2Y1 or P2X7 expression was suppressed with shRNAs, or enhanced with expression of P2Y13 or P2X7-GFP (Fig. 5C-F).

Neurons were first treated with ADP and the adenylate cyclase inhibitor (SQ-22536), which attenuated ADP-mediated axon elongation (152.54 $\pm$ 4.43  $\mu\text{m}$  vs 367.85 $\pm$ 10.69  $\mu\text{m}$  in neurons treated with ADP alone) and reduced axon growth to below control values (189.19 $\pm$ 5.57  $\mu\text{m}$  in non-treated neurons; Fig. 5A,B). The reduction of axon growth in response to adenylate cyclase inhibition (134.25 $\pm$ 3.49  $\mu\text{m}$ ) was similar to that observed following P2Y1 inhibition with MRS-2179 (146.64 $\pm$ 6.63  $\mu\text{m}$ ), although no further decrease in axon growth was observed following co-administration of SQ-22536 and MRS-2179 (137.50 $\pm$ 6.66  $\mu\text{m}$ ). SQ-22536 administration failed to reduce axon growth in the presence of dbcAMP (298.30 $\pm$ 12.39  $\mu\text{m}$  vs 322.72 $\pm$ 15.42  $\mu\text{m}$  in neurons treated with dbcAMP alone) and furthermore, inhibition of adenylate cyclase blocked axon elongation when P2Y13 was inhibited with MRS-2211 (150.88 $\pm$ 5.07  $\mu\text{m}$  vs 362.01 $\pm$ 10.75  $\mu\text{m}$ ). The inhibitory effect of MRS-2179 (128.69 $\pm$ 4.67  $\mu\text{m}$  vs 156.55 $\pm$ 5.25  $\mu\text{m}$  in control neurons) was reversed by co-administration of 5  $\mu\text{M}$  forskolin (199.06 $\pm$ 6.80  $\mu\text{m}$ ). Similarly, forskolin reversed the inhibition of axon elongation provoked by P2Y1 shRNA or exogenous P2Y13 expression (Fig. 5C,E). Inhibition of phosphodiesterase 4 induced axon elongation in the presence of the P2Y1 antagonist MRS-2179 (337.90 $\pm$ 10.84  $\mu\text{m}$  vs 138.06 $\pm$ 3.57  $\mu\text{m}$ ; Fig. 5B) and in neurons expressing exogenous P2Y13 (328.02 $\pm$ 10.79  $\mu\text{m}$  vs 131.44 $\pm$ 4.81  $\mu\text{m}$ ; Fig. 5E), in both cases above that of their respective control neurons (180.25 $\pm$ 3.89  $\mu\text{m}$  or 193.67 $\pm$ 6.00  $\mu\text{m}$ ). Treatment with the cAMP antagonist, cAMPS-Rp (20  $\mu\text{M}$ ), attenuated the increase in axon length produced by ADP or inhibiting P2Y13 with MRS-2211 (Fig. 5A,B).

The axon elongation provoked by suppressing P2X7 was abolished by the adenylate cyclase antagonist SQ-22536 and by the cAMP antagonist cAMPS-Rp. Furthermore, the inhibitory effect of P2X7-GFP expression on axonal elongation was reversed by the administration of forskolin or the PDE4 inhibitor (Fig. 5D,F). Taken together, these results demonstrate that P2Y1 and P2Y13 receptors exert opposing regulatory effects on adenylate cyclase activity, and that P2X7 negatively regulates adenylate cyclase activity. These findings raise the question of whether the coordinated activity of these three receptors and their extracellular agonists can together modulate axon elongation through a common pathway involving adenylate cyclase, cAMP and PKA.

#### Adenylate cyclase 5 coordinates the regulation of axon elongation mediated by P2Y1, P2Y13 and P2X7

All six mentioned adenylate cyclases (AC1, 3, 5, 6, 8 and 9) can be activated by Gq or inhibited by Gi proteins, but only AC5 and AC6 are inhibited by sub-micromolar concentrations (0.2–0.6  $\mu\text{M}$ ) of  $\text{Ca}^{2+}$  (Cooper, 2003; Guillou et al., 1999), such as those produced by  $\text{Ca}^{2+}$  entry through the ATP membrane receptor P2X7 (Leon et al., 2006). However, AC5 is also

activated by PKC $\zeta$  (Kawabe et al., 1994), which in turn has been implicated in axon establishment (Schwamborn and Puschel, 2004) and binds to G $\alpha_q$  proteins (Garcia-Hoz et al., 2010). In fact, PKC $\zeta$  inhibition did significantly impair axonal elongation mediated by ADP or BBG (Fig. 6A,D). The axon elongation produced by MRS-2211 (265.11 $\pm$ 10.77  $\mu\text{m}$  vs 141.71 $\pm$ 3.59  $\mu\text{m}$  in control neurons) was also blocked by PKC $\zeta$  inhibition (125.73 $\pm$ 5.71  $\mu\text{m}$ ; Fig. 6A,D). However, PKC $\zeta$  inhibition did not reduce axonal elongation when adenylate cyclases were activated by forskolin (Fig. 6D). These data demonstrate that PKC $\zeta$  mediates an adenylate-cyclase-dependent mechanism triggered by ADP, which can be negatively regulated by P2Y13 and P2X7 receptor activation.

Thus, we investigated the possibility that the AC5 adenylate cyclase isoform co-ordinates the activities of P2Y1, P2Y13 and P2X7 receptors during axon elongation.

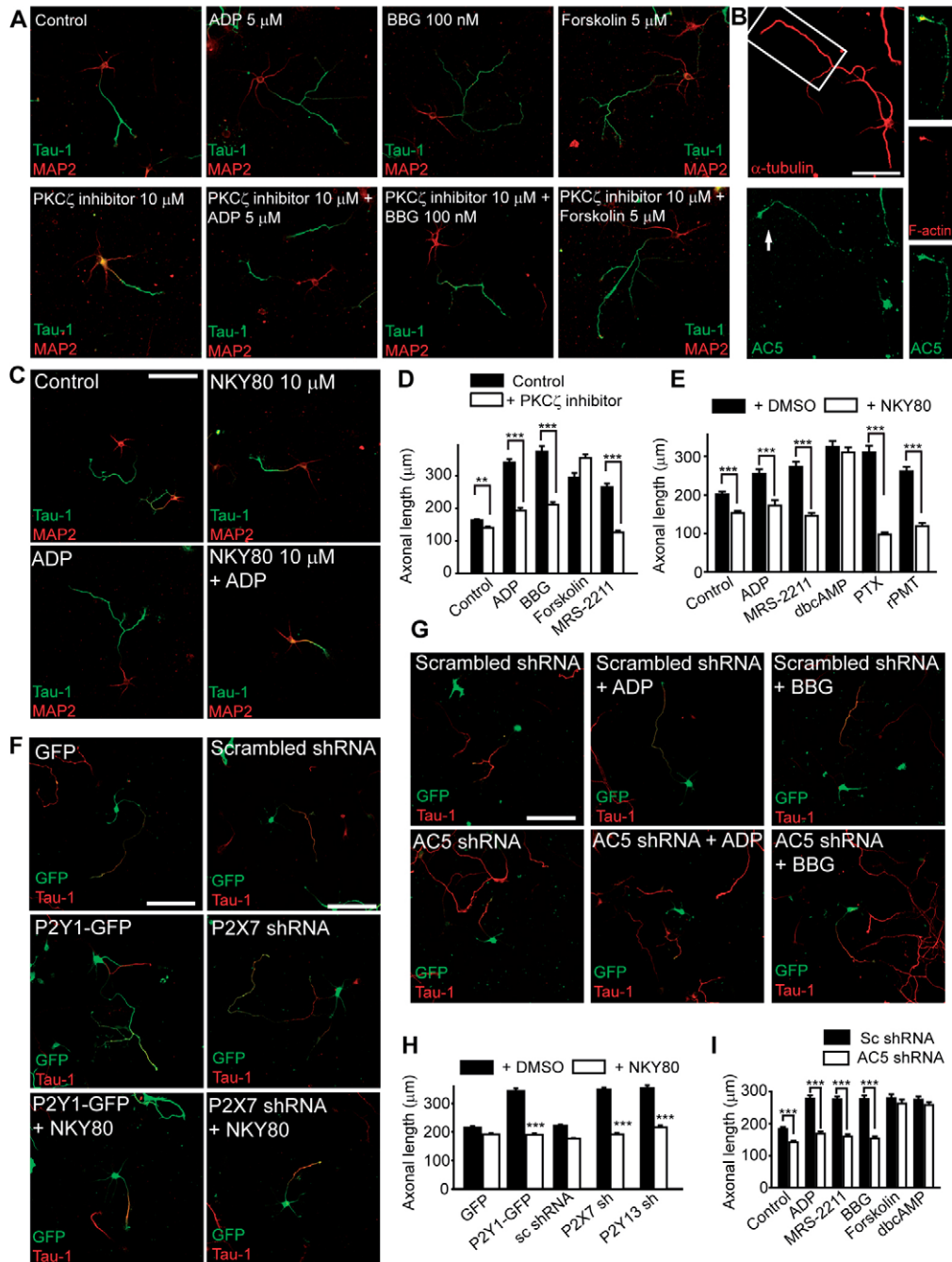
In hippocampal neurons, AC5 was found in the soma, as well as in a distal gradient in the axon and the actin region of the growth cone, and to a lesser extent in minor neurites, similar to the receptors P2Y1, P2Y13 and P2X7 (Fig. 6B; Fig. 1) (Diaz-Hernandez et al., 2008). NKY80 (10  $\mu\text{M}$ ) is a highly selective inhibitor of AC5 (Onda et al., 2001) that impaired ADP-mediated axon elongation in hippocampal neurons at 1 to 3 DIV (170.92 $\pm$ 14.04  $\mu\text{m}$  vs 253.38 $\pm$ 11.48  $\mu\text{m}$  in ADP-treated neurons vs 201.34 $\pm$ 7.62  $\mu\text{m}$  in control neurons; Fig. 6C,E). Similarly, axon elongation following P2Y13 inhibition with MRS-2211 was abolished by NKY80, although it had no effect on axon elongation in neurons treated with 2 mM dbcAMP (Fig. 6E). Moreover, axon growth mediated by Gq activation (with rPMT) or Gi inhibition (with pertussis toxin) was impaired by AC5 inhibition (Fig. 6E).

Next, neurons were nucleofected with plasmids expressing P2Y1-GFP, or interference shRNAs for P2X7 or P2Y13 and treated with NKY80 at 1 to 3 DIV (Fig. 6F,H). Although P2Y1-GFP expression promoted axon elongation, this effect was impaired by inhibiting AC5 with NKY80. Similarly, NKY80 attenuated the increases in axon elongation observed in neurons expressing P2X7 or P2Y13 shRNAs.

Nucleofection of neurons with AC5 interference shRNAs produced similar effects to the pharmacological inhibition of AC5 (Fig. 6G,I). Axon elongation was reduced in neurons expressing AC5 shRNA (141.54 $\pm$ 4.68  $\mu\text{m}$  vs 183.35 $\pm$ 6.06  $\mu\text{m}$  in those expressing scrambled shRNA), and the increase in axon elongation produced by ADP (275.36 $\pm$ 9.55  $\mu\text{m}$ ), MRS-2211 (278.13 $\pm$ 9.15  $\mu\text{m}$ ) and BBG (275.96 $\pm$ 11.01  $\mu\text{m}$ ) treatment in scrambled-shRNA-nucleofected neurons were abrogated in neurons expressing AC5 shRNA (160.06 $\pm$ 6.59, 168.31 $\pm$ 7.24 and 153.65 $\pm$ 6.91  $\mu\text{m}$ , respectively). AC5 shRNA expression failed to reduce axonal elongation in neurons treated with forskolin or dbcAMP (Fig. 6I).

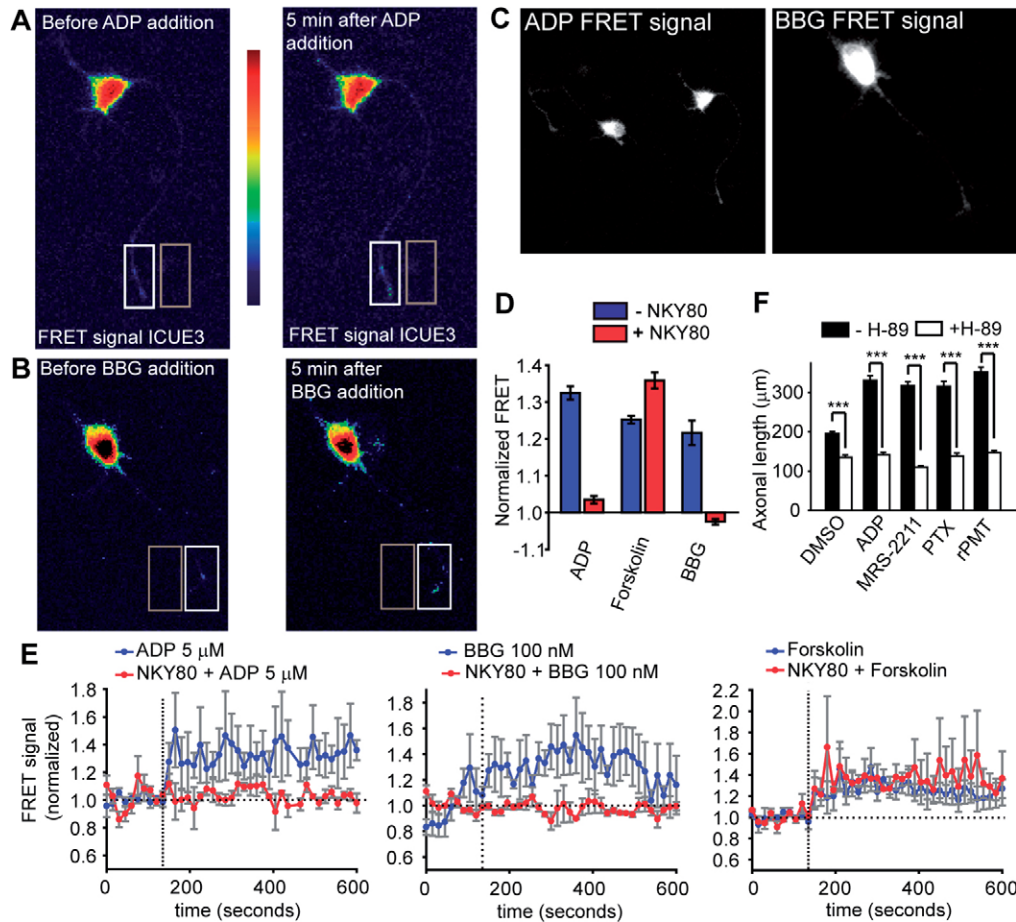
Finally, neurons were nucleofected with a plasmid expressing a FRET-based biosensor to detect cAMP levels (ICUE3) (DiPilato and Zhang, 2009). Neurons were treated with ADP (5  $\mu\text{M}$ ) or the P2X7 antagonist BBG (100 nM), alone or in the presence of NKY80 (10  $\mu\text{M}$ ), which was added 30 minutes before treatment (Fig. 7). Both ADP and BBG increased by 20–30% cAMP levels in the distal region of the axon (Fig. 7D,E) compared with the normalized value before treatment (Fig. 7E). These increases were abolished by pre-treatment with NKY80. As a control, neurons were treated with forskolin in the presence or absence of NKY80. In both cases cAMP levels were similar to





**Fig. 6. Adenylate cyclase 5 activity is required for proper axonal elongation in response to ADP or P2X7 inhibition.** (A,D) Hippocampal neurons cultured from day 1 to day 3 in vitro in the presence or absence of the PKC $\zeta$  inhibitor, PKC $\zeta$  pseudosubstrate (10  $\mu$ M) in combination with ADP 5  $\mu$ M, BBG 100 nM, MRS-2211 5  $\mu$ M or forskolin 5  $\mu$ M. Neurons were stained with anti-MAP2 and anti-Tau-1 antibodies. Graph in D shows the mean axonal lengths  $\pm$  s.e.m. from three independent experiments. (B) Distribution of adenylate cyclase 5 in hippocampal neurons at 3 DIV. Arrow indicates the AC5 in the distal region of the axon. Right panels show the distal region of the axon stained for AC5 and F-actin. Scale bar: 100  $\mu$ m. (C) Hippocampal neurons treated from day 1 to day 3 in vitro with ADP (5  $\mu$ M) in the presence or absence of the adenylate cyclase 5 inhibitor NKY80 (10  $\mu$ M). Scale bar: 100  $\mu$ m. (E) Mean axon lengths  $\pm$  s.e.m. of 3 DIV neurons treated with vehicle (black bars) or NKY80 (white bars), in combination with ADP (5  $\mu$ M), the P2Y13 antagonist MRS-2211, dbcAMP (2 mM), rPMT or PTX. Note that addition of dbcAMP impaired the inhibitory effect of NKY80 on axon growth; \*\*\* $P$ <0.001. Data are from three independent experiments analyzing 100 neurons for each condition in each experiment. (F,H) Neurons nucleofected with GFP, P2Y1-GFP, scrambled shRNA, P2X7 shRNA or P2Y13 shRNA were cultured from day 1 to day 3 in vitro with vehicle or the adenylate cyclase 5 inhibitor, NKY80. (F) Representative images of these neurons. (H) Mean axonal lengths  $\pm$  s.e.m. from three independent experiments analyzing 100 neurons for each condition in each experiment; \*\*\* $P$ <0.001. (G,I) Neurons were nucleofected with scrambled shRNA or AC5 shRNA. (G) Representative images of 3 DIV neurons. (I) Mean axon length  $\pm$  s.e.m. of neurons shown in G cultured in the presence of the indicated compounds at the concentrations shown previously. Scale bars: 100  $\mu$ m.





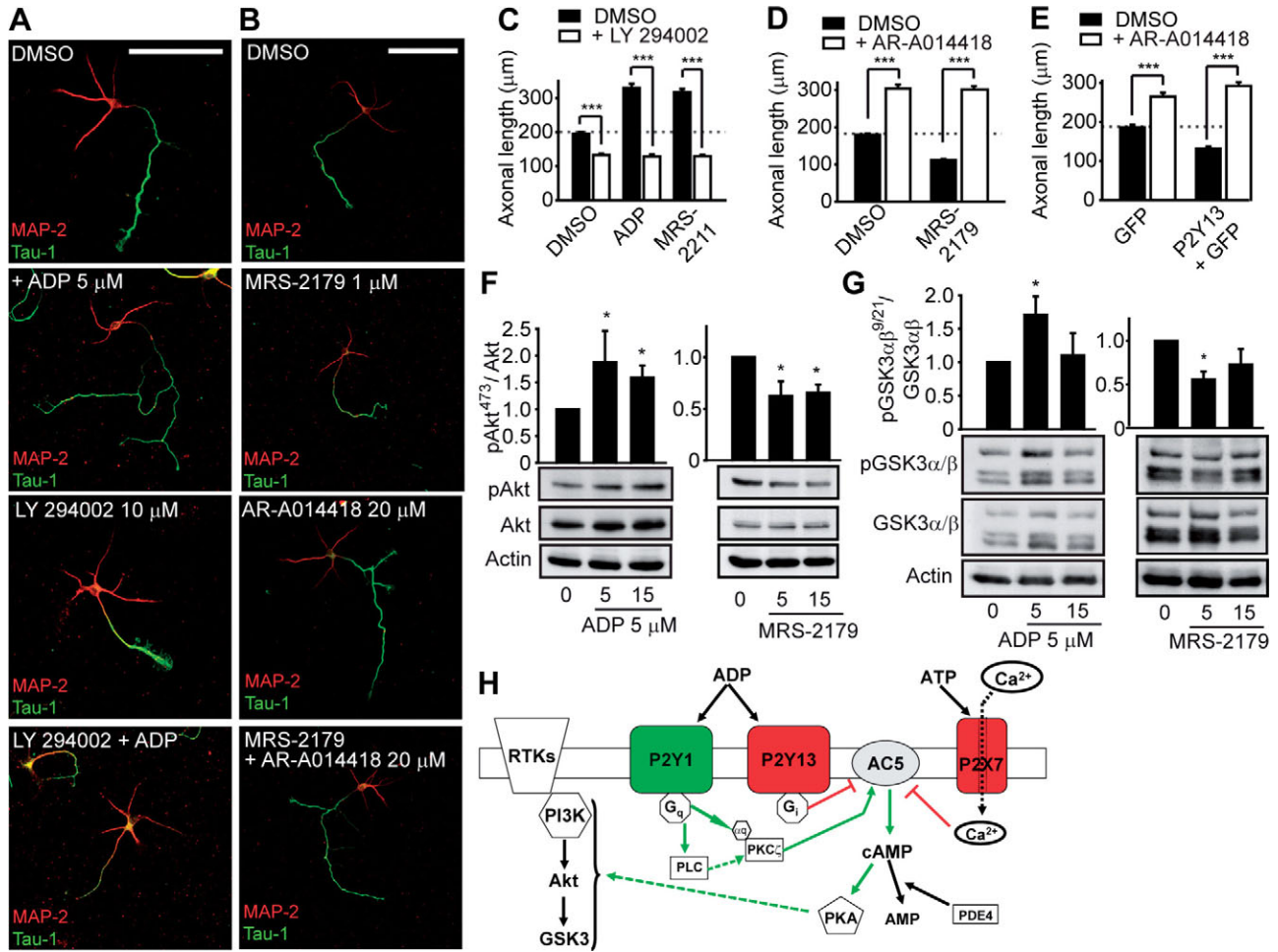
**Fig. 7. AC5 inhibition impairs the increase in cAMP levels at the distal region of the axon generated by ADP or BBG.** (A–C) Hippocampal neurons nucleofected with the FRET biosensor ICUE3. After 2 DIV, neurons were treated with ADP or BBG and processed as described in the Materials and Methods. Pseudocolor images show FRET signals obtained from 2 DIV neurons before or after addition of ADP for 5 minutes. Pseudocolor images were obtained using Adobe Photoshop CS4 software. The white rectangles indicate the area measured and the grey rectangles indicate the area of background measured. Grayscale images in C show the FRET signal obtained after 5 minutes treatment with ADP or BBG. (D) Mean ( $\pm$  s.e.m.) FRET signal (YFP/CFP) from four different neurons treated as described in the Materials and Methods section with the compounds indicated in the figure. (E) Mean ( $\pm$  s.e.m.) FRET signal obtained every 15 seconds during the treatment with ADP, BBG or forskolin in the presence or absence of NKY80 normalized to the base line. Dashed lines indicate the time of treatment with ADP, forskolin or BBG, and the normalized base line. (F) Mean axonal length of 3 DIV neurons treated from day 1 to day 3 in vitro with ADP, MRS-2211, rPMT or PTX, alone (black bars) or in combination with the PKA inhibitor, H-89 (white bars). Data are the means  $\pm$  s.e.m. of three independent experiments.

those observed following exposure to ADP or BBG. Moreover, inhibition of PKA, a cAMP-dependent kinase, abolished the increase in axon elongation produced by ADP or MRS-2211 (Fig. 7F). Taken together, these results demonstrate that adenylate cyclase 5 is a common mediator of the axon elongation regulated by P2Y1, P2Y13 and P2X7 receptors in response to extracellular ADP and ATP.

#### ADP regulates the PI3K pathway

Initial axon establishment was not impaired by P2Y1 shRNA, P2X7–GFP, P2Y13 expression or AC5 shRNA nucleofection, suggesting that these purinergic receptors act through a common signaling pathway to specifically regulate axon elongation. Enhanced axon growth following P2X7 inhibition appears to be mediated through the phosphoinositide 3-kinase (PI3K)–Akt–GSK3 pathway (Diaz-Hernandez et al., 2008) and, moreover, P2Y13 activity can regulate GSK3 phosphorylation (Ortega et al.,

2008). Thus, we examined whether the effects of ADP in axon elongation were mediated by the PI3K pathway. In 3 DIV hippocampal neurons, Akt and GSK3 phosphorylation was augmented following acute administration of ADP (5  $\mu$ M; Fig. 8F,G). When PI3K was inhibited by the presence of LY-294002 (10  $\mu$ M) at 1 to 3 DIV, the axon elongation induced by ADP or MRS-2211 was suppressed in both cases (Fig. 8A,C). Hippocampal neurons expressing the P2Y13 receptor or treated with the P2Y1 antagonist, MRS-2179, were cultured from day 1 to day 3 in vitro in the presence of a GSK3 inhibitor (AR-A014418; 20  $\mu$ M). The inhibition of GSK3 reversed the inhibitory effects of both P2Y1 inhibition and P2Y13 expression on axon elongation (Fig. 8B,D,E). Moreover, P2Y1 inhibition diminished the phosphorylation of Akt and GSK3 (Fig. 8F,G). All together, these results suggest a regulation of adenylate cyclase 5 by ADP, ATP, P2Y1, P2Y13 and P2X7 receptors, through G proteins and extracellular  $Ca^{2+}$  entry. This modulates cAMP levels and PKA function, regulating the input



**Fig. 8. The axon-elongating effects of ADP are mediated by the PI3K–Akt–GSK3 pathway.** (A,B) Hippocampal neurons cultured from day 1 to day 3 in vitro in the presence of vehicle, the PI3K inhibitor LY-294002 (A) or the GSK3 inhibitor AR-014418 (B) in combination with ADP, antagonists of P2Y13 (MRS-2211) or P2Y1 (MRS-2179) or following nucleofection with the P2Y13 receptor. (C–E) Mean axonal lengths  $\pm$  s.e.m. of neurons shown in A and B. Axons were identified as Tau-1-positive processes. Data are from three independent experiments analyzing at least 100 neurons for each condition in each experiment; \*\*\* $P < 0.001$ . (F,G) Quantification of Akt and GSK3 phosphorylation in extracts of hippocampal neurons cultures treated with ADP (5  $\mu$ M) or the P2Y1 antagonist MRS-2179, for 5 or 15 minutes. Data are the means  $\pm$  s.e.m. of three independent experiments; \* $P < 0.05$ . Representative western blots from each experiment are shown below the graphs. Actin was used as a loading control. (H) Proposed model of the intracellular mechanisms that coordinate the action of P2Y1, P2Y13 and P2X7 receptors in regulating axon elongation.

of purinergic receptors signaling on the PI3K–Akt–GSK3 pathway (Fig. 8H).

## Discussion

Purines and purinergic receptors have been implicated in a variety of physiological and pathological conditions, including neurotransmission, brain development, inflammation, pain, central nervous system injury, neuropsychiatric disorders and neurodegenerative diseases (Burnstock, 2007; Burnstock, 2008). Purinergic receptors have been identified at early stages of brain developmental when they regulate stem cell proliferation (Burnstock and Ulrich, 2011). However, their role in the development of neuronal morphology and axon growth remains largely unknown. We previously demonstrated that ATP, acting at the purinergic P2X7 receptor, negatively modulates axon growth, generating an increase in calcium at the distal axon (Diaz-Hernandez et al., 2008), whereas inhibition or suppression of this receptor promotes axon elongation. The present study

demonstrates that ADP enhances axon growth in cultured hippocampal neurons through the activation of the metabotropic P2Y1 receptor. Both ADP and P2Y1 have been previously described as regulators of neurosphere proliferation (Mishra et al., 2006). P2Y1 is also necessary for proper migration of intermediate neuronal progenitors to the neocortical subventricular zone (Liu et al., 2008). Our results show that P2Y1 function is necessary for proper axonal elongation, which can be downregulated by the action of ADP at the P2Y13 receptor or the activation of the P2X7 receptor. The overall positive results in axon elongation promoted by ADP might be the result of the higher expression of P2Y1 than P2Y13 receptors, as shown in supplementary material Fig. S1. The contrasting effects of P2Y1 and P2Y13 activation on axon elongation are consistent with their opposing roles in the control of pain or insulin secretion (Amisten et al., 2010; Malin and Molliver, 2010). Both P2Y1 and P2Y13 are expressed in the distal region of the axon, similar to P2X7 (Diaz-Hernandez et al., 2008). Thus,

in both normal and pathological conditions, variations in P2Y1, P2Y13 or P2X7 membrane expression, or extracellular ATP and ADP concentrations, can modulate axon elongation. In this sense, the different ectonucleotidase families that modify the number of phosphates in adenine nucleotides could be important regulators (Langer et al., 2008). For example, expression in cultured neurons of tissue non-specific alkaline phosphatase (TNAP), which reduces extracellular ATP levels, improves axonal growth (Diez-Zaera et al., 2011). Our results suggest that axon elongation is regulated through the coordination of the ionotropic ATP-gated P2X7 receptor and ADP-activated P2Y receptors. Thus, in the absence of elevated concentrations of extracellular ATP, P2Y1 receptors can potentiate axonal elongation, and this action might be partially controlled by P2Y13 receptors and P2X7 receptors. However, after different kinds of 'acute' CNS injury (e.g. ischemia, hypoxia, mechanical stress, axotomy), extracellular ATP can reach high concentrations, up to the millimolar range, flowing out from cells into the extracellular space, exocytotically, by transmembrane transport, or as a result of cell damage (Franke and Illes, 2006). In that case, P2X7 activation by high concentrations of extracellular ATP can negatively modulate axonal elongation. In the case of acute spinal cord injury, P2X7 inhibition substantially improves functional recovery and diminished cell death in the peritraumatic zone (Wang et al., 2004). Moreover, P2 purinergic receptors are also involved in neurodegenerative diseases. In the case of Alzheimer disease, P2X7 receptor expression is upregulated (Parvathenani et al., 2003) and P2Y1 receptor expression shows an altered distribution in human AD brain (Moore et al., 2000).

The modulation of axon elongation by three distinct purinergic receptors and varying concentrations of their agonists suggests that their regulatory effects are coordinated at a common intracellular checkpoint. cAMP is a second messenger with regulatory effects on axon formation and elongation (Shelly et al., 2010). Adenylate cyclase is implicated in multiple pathways that modulate axonal growth, both positively and negatively, in developmental and pathological conditions. For example, elevation of intracellular cAMP levels by dbcAMP or inhibition of PDE4, can overcome myelin inhibition both *in vitro* and *in vivo* (Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004).

Our results show that P2Y1 activation and P2X7 inhibition both increase cAMP levels at the distal region of the axon. One adenylate cyclase isoform, AC5, is regulated both by G proteins (Willoughby and Cooper, 2007) and submicromolar concentrations of  $Ca^{2+}$  (Cooper, 2003; Guillou et al., 1999). Inhibition of AC5 blocks the axon elongation promoted by activation of P2Y1 receptor or Gq, or inhibition of Gi proteins, and it impairs the axon elongation produced by ADP or P2X7 inhibition. Gq proteins activate phospholipase C (PLC) that in turn can activate PKCs, including PKC $\zeta$  (van Dijk et al., 1997). Moreover, PKC $\zeta$  associates to Gq upon G-protein-coupled receptor activation (Garcia-Hoz et al., 2010). This activation of PKC $\zeta$  can stimulate AC5 activity (Kawabe et al., 1994; Willoughby and Cooper, 2007). Indeed, we demonstrate that inhibition of PKC $\zeta$  impairs the axon elongation produced by ADP, P2Y13 inhibition or P2X7 inhibition. The reduction in axonal elongation produced by PKC $\zeta$  inhibition is avoided by adenylate cyclase activation, placing PKC $\zeta$  upstream of adenylate cyclase 5, and being activated by the P2Y1 receptor. The activation of AC5 by PKC $\zeta$  is, in turn, regulated in the

opposite way by P2Y13, Gi and P2X7. In the case of P2X7 inhibition, retinoic-acid-induced differentiation of N2a cells has been shown to decrease P2X7 receptor levels (Wu et al., 2009) but also, to increase AC5 mRNA levels during P19 cell differentiation (Lipskaia et al., 1997). AC5 mRNA is expressed in the striatum and hippocampus of the adult brain (Kheirbek et al., 2009), and it increases in the hippocampus during post-natal development up to day 14 (Matsuoka et al., 1997). Studies in AC5 knockout mice demonstrate that AC5 is involved in a signaling pathway in corticostriatal plasticity and striatum-dependent learning. Moreover, loss of AC5 compromises the ability of both contextual and discrete cues to modulate instrumental behavior (Kheirbek et al., 2010; Kheirbek et al., 2009), and the AC5 knockout mice show striking anxiolytic and antidepressant phenotypes in standard behavioral assays (Krishnan et al., 2008). Finally, AC5-knockout mice have markedly attenuated pain-like responses in neuropathic pain models (Kim et al., 2007), in accordance with the proposed roles of P2Y1 and P2Y13 in pain (Malin and Molliver, 2010). Our results demonstrate that AC5 protein is found in the soma and shows an increasing distal gradient along the axon and axonal growth cone. This distribution of AC5 in the axon mirrored the expression of the three purinergic receptors of interest in hippocampal neurons, and the localization of adenylate cyclase (Mizumashi et al., 2001) and PKA (Sato et al., 2002).

Signaling through each of the three purinergic receptors can enhance or diminish the velocity of axon growth, but in no case was axon formation impaired. The co-ordination of these signaling pathways by adenylate cyclase 5 could be a key means to regulate a main pathway involved in axon growth and neuronal connectivity. Taken together with our previous findings, our results indicate that activation of P2Y1 potentiates the PI3K–Akt–GSK3 pathway to promote axon elongation, whereas ATP exerts an opposing effect, downregulating the signaling through this pathway and inhibiting axon growth. ADP activation of P2Y1 increases Akt and GSK3 phosphorylation while promoting axon elongation, an effect previously demonstrated following P2X7 inhibition (Diaz-Hernandez et al., 2008). This enhanced elongation can be impaired by inhibiting PI3K, whereas the attenuation of axon growth by P2Y1 inhibition can be counteracted by inhibiting GSK3, and such inhibition has been previously shown to induce axonal elongation (Garrido et al., 2007). Further studies will be necessary to fully elucidate the complex signaling network that unites adenylate cyclase 5, cAMP and PKA, and the PI3K–Akt–GSK3 pathway. The complex coordination of purines and purinergic receptors described in our work do not exclude that other parallel signaling pathways regulated by these receptors might also be involved in the regulation of neuronal development and function. However, on the basis of the present findings, we can propose a model whereby two purine nucleotides, ATP and ADP, control the activity of three purinergic receptors, P2Y1, P2Y13 and P2X7, in response to changes in extracellular purine concentrations in both normal and pathological conditions. These interactions positively or negatively influence adenylate cyclase 5 activity, thereby modulating the capacity of PKA and neurotrophic factors to activate the PI3K–Akt–GSK3 pathway. Accordingly, the development of P2Y1-specific agonists, combined with specific antagonists of P2X7 and P2Y13, might provide an efficient means of treating the brain diseases associated with the reduction of synaptic contacts, neuronal



death or axonal degeneration, by promoting the arrival of axons and new synapse generation.

## Materials and Methods

### Reagents

The following reagents were used in this study (catalog nos are given in parentheses): ATP from Roche, ADP (A2754), 2meSADP (M152), forskolin (F6886), H89 (B1427) and NKY80 (N2165), all from Sigma-Aldrich; MRS-2211 (2402), cAMPS-Rp (1337) and MRS-2179 (0900) from Tocris; pertussis toxin (G100-0050) from Biomol; *Pasteurella multocida* toxin, rPMT (512743), U-73122 (662035), SQ-22536 (568500), phosphodiesterase 4 inhibitor (3,5-dimethyl-1-(3-nitrophenyl)-1H-pyrazole-4-carboxylic acid ethyl ester, 524717), LY-2940002 (440202), AR-A014418 (361549) and PKC pseudosubstrate inhibitor, myristoylated (539624), all from Calbiochem. Commercial antibodies against the following proteins were used: P2Y1 and P2Y13 (Alomone, Jerusalem, Israel); P2X7 (Abcam); Akt (Santa Cruz Biotechnology);  $\alpha$ -tubulin and  $\beta$ -actin (Sigma); phosphorylated GSK (at amino acid S9/S21), phosphorylated Akt (at amino acid S473) and GADPH (Cell Signaling); GSK3 (Invitrogen-Biosource); anti-adenylate cyclase 5 (Abcam); and Tau-1 and MAP2 (Millipore). Oligonucleotides for quantitative RT-PCR were obtained from Applied Biosystems.

### Cell culture

Hippocampal neuronal cultures were prepared as described previously (Banker and Goslin, 1988). Briefly, the hippocampus was removed from E17 mouse embryos and after dissection and washing three times in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Hank's balanced salt solution (HBSS), the tissue was digested in the same solution containing 0.25% trypsin for 15 minutes at 37°C. The hippocampi were then washed again three times in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free HBSS and dissociated with a fire-polished Pasteur pipette. The cells were counted, resuspended in plating medium (MEM, 10% horse serum, 0.6% glucose) and plated at a density of 5000/cm<sup>2</sup> on polylysine-coated coverslips (1 mg/ml). Neurons were incubated at 37°C for 2 hours before switching them to neuronal culture medium (Neurobasal, B-27, glutamax-1). To analyze the effect of P2Y receptor agonists and antagonists, the compounds were added to the cultured neurons 1 day after plating at the concentrations indicated, maintaining them for a further 48 hours. For biochemical experiments hippocampal neurons were plated at a density of 200,000/cm<sup>2</sup> on 60 mm plates coated with polylysine (0.5 mg/ml). Before plating, the different plasmids were nucleofected into the neurons using the Amaxa nucleofection kit according to the manufacturer's instructions for hippocampal neurons. Only scattered glial cells appeared after 3 DIV, and our neuronal cultures were 99% pure.

HEK-293T cells were maintained in DMEM (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS). The cells were resuspended 1 day before transfection, and plated at a density of 10<sup>5</sup> cells/cm<sup>2</sup>, and they were maintained in medium containing 0.5% FCS. HEK-293T cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

### Plasmids

Full-length human P2Y1 cDNA (cDNA clone number MGC: BC074784; Geneservice Ltd) was subcloned into the *EcoRI* and *BamHI* sites of the mammalian pEGFP-N1 expression vector after PCR amplification with the primers: 5'-CTAGGAATTCATGACCGAGGTCTGTGGCC-3' and 3'-CTAGGGATCCGGCAGGCTTGTATCTCCATTCT-5'. The full-length human P2Y13 cDNA was purchased from Open Biosystems (cDNA clone number: BC041116). P2Y1 and P2Y13 receptor knockdown was achieved using RNA interference (RNAi), applying a vector-based shRNA approach. The shRNA target sequences 5'-GCTGTGTCTTACATCCCTTTC-3' or 5'-GCATCTCCGTGTAC-ATGTTCA-3' were selected for P2Y1, and 5'-CCTTCCGACTCACACCTT-3' or 5'-CAGCTGTTTATTGCTAAA-3' for the P2Y13 receptor, in accordance with a previously reported rational design protocol (Reynolds et al., 2004). The P2X7 expression plasmids and shRNA used here have been described previously (Diaz-Hernandez et al., 2008). As a control we used the firefly luciferase-targeted oligonucleotide, 5'-CTGACGCGGAATACTTTCGA-3'. Synthetic forward and reverse 64-nucleotide oligonucleotides (Sigma Genosys) were designed, annealed and inserted into the *BglIII-HindIII* sites of the pSUPER.neo.GFP vector (OligoEngine, Seattle, WA) following the manufacturer's instructions. Nucleofected neurons were identified by the expression of green fluorescent protein (GFP) from this vector. The adenylylase cyclase 5 interference shRNAs (79 and 84) and control scrambled shRNA were purchased from Origene (TG506651).

### FRET imaging and analysis

Hippocampal neurons were nucleofected with a plasmid expressing the FRET biosensor ICUE3 (DiPilato and Zhang, 2009). Neurons were cultured for 2 DIV and examined with a C9100-02 CCD camera (Hamamatsu) and an Axiovert200 Zeiss microscope, using a 75W/2 Xenon XBO lamp and a 40 × 1.3 NA objective

(Zeiss). The excitation wavelength used was 422–432 nm, and emission wavelengths were separated with a double dichroic filter (440–500 nm and 510–610 nm), with 460–500 nm and 528.5–555.5 nm emission filters for CFP and YFP fluorescence, respectively. Images were collected and analyzed with Metamorph 7.1 r2 software (Universal Imaging) and live images were acquired for 120–140 msec/frames at 15-second intervals. For global manipulation of cAMP signaling, pharmacological agents were applied to the bath after 150 seconds of baseline recording. The intensity of the CFP and YFP fluorescence was measured at the distal region of the axon of hippocampal neurons using ImageJ software. For the ratiometric FRET analysis, the background was subtracted from the CFP and YFP signals of the defined distal region of the axon (background intensity was calculated from a cell-free region using ImageJ software), which were then normalized to the control value (averaged over 150 seconds of baseline recording), and the FRET value was calculated as the ratio of the YFP:CFP signal. Neuronal medium was switched to Neurobasal medium without Phenol Red 30 minutes prior to analysis. The concentrations of pharmacological agents applied to the bath were as follows: ADP, 5  $\mu\text{M}$ ; BBG, 100 nM; and forskolin, 5  $\mu\text{M}$ . These treatments were applied either alone or following a 30-minute preincubation with NKY80 (10  $\mu\text{M}$ ) prior to image acquisition, and the compounds tested remained present throughout the experiment.

### Immunocytochemistry

Neurons were cultured for 3 DIV followed by fixation in 4% paraformaldehyde for 20 minutes. Non-specific binding was blocked with 0.22% gelatin and 0.1% Triton X-100 in 0.1 M phosphate buffer. Cells were then incubated with primary antibodies for 1 hour at room temperature, washed and incubated with Alexa-Fluor-conjugated secondary antibodies (1:1000) and Alexa-Fluor-594-conjugated phalloidin (1:100). The coverslips were mounted using Fluoromount G (Southern Biotech) and images acquired on a LSM510 confocal microscope coupled to an Axiovert 200M (Zeiss) microscope. Axon length and ramifications was analyzed with the NeuronJ program and the fluorescence intensity was evaluated using the RGB color profiler tool of the ImageJ software. Images were processed and presented using Adobe Photoshop and Illustrator CS3.

### Western blotting

Cultured neurons were lysed and homogenized in a buffer containing: 20 mM Hepes pH 7.4, 100 mM NaCl, 10 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM EDTA and protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin and 10  $\mu\text{g}/\text{ml}$  pepstatin). Proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The experiments were performed using primary polyclonal antisera (and dilutions) against: P2Y1 (1:50); P2Y13 (1:50); Akt (1:1000); pS473Akt (1:1000); pS9/21GSK-3 (1:1000) or monoclonal antibodies against  $\alpha$ -tubulin (1:1000); GSK-3  $\alpha/\beta$  (1:1000) and  $\beta$ -actin (1:1000). The membranes were probed overnight at 4°C with the selected antibodies in 5% BSA and then with a secondary goat anti-mouse monoclonal antibody or goat anti-rabbit polyclonal antiserum (both at 1:5000; Amersham) to detect the primary antibodies. Antibody binding was visualized by ECL (Amersham).

### Statistics

All experiments were repeated at least three times and the results are presented as the means  $\pm$  s.e.m. or as a box-plot showing the distribution of axonal length of all neurons from at least three experiments. Axon length was quantified in at least 100 neurons for each condition and experiment, and all axons were identified as Tau-1-positive processes. Statistical differences were determined by ANOVA using the SigmaPlot software.

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