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BRIEF COMMUNICATION

$P2Y_{12}$ receptor expression is a critical determinant of functional responsiveness to ATX's MORFO domain

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Abstract In the central nervous system, the formation of the myelin sheath and the differentiation of the myelinating cells, namely oligodendrocytes, are regulated by complex signaling networks that involve purinergic receptors and the extracellular matrix. However, the exact nature of the molecular interactions underlying these networks still needs to be defined. In this respect, the data presented here reveal a signaling mechanism that is characterized by an interaction between the purinergic $P2Y_{12}$ receptor and the matricellular extracellular matrix protein autotaxin (ATX), also known as ENPP2, phosphodiesterase-Iα/ATX, or lysoPLD. ATX has been previously described by us to mediate intermediate states of oligodendrocyte adhesion and to enable changes in oligodendrocyte morphology that are thought to be crucial for the formation of a fully functional myelin sheath. This functional property of ATX is mediated by ATX's modulator of oligodendrocyte remodeling and focal adhesion organization (MORFO) domain. Here, we show that the expression of the $P2Y_{12}$ receptor is necessary for ATX's MORFO domain to exert its effects on differentiating oligodendrocytes. In addition, our data demonstrate that exogenous expression of the $P2Y_{12}$ receptor can render cells responsive to the known effects of ATX's MORFO domain, and they identify Rac1 as an intracellular factor mediating the effect of ATX-MORFO-$P2Y_{12}$ signaling on the assembly of focal adhesions. Our data further support the idea that a physical interaction between ATX and the $P2Y_{12}$ receptor provides the basis for an ATX-MORFO-$P2Y_{12}$ signaling axis that is crucial for mediating cellular states of intermediate adhesion and morphological/structural plasticity.

Keywords Oligodendrocyte · Autotaxin · Purinergic receptor · Adhesion · $P2Y_{12}$

Introduction

Signaling involving purinergic receptors has emerged as an essential mechanism regulating cell–cell communication in the central nervous system (CNS) [1–5]. First described to mediate synaptic transmission via direct neuron–neuron communication, purinergic receptor signaling is now recognized to be equally important for neuron–glia and glia–glia interactions including those that control the stepwise differentiation of the myelinating cells of the CNS, namely oligodendrocytes. As a first step during oligodendrocyte differentiation, migratory bipolar oligodendrocyte progenitor cells differentiate into post-migratory oligodendrocytes that extend several branched processes. These post-migratory oligodendrocytes then mature into cells with a complex and expanded process network to ultimately differentiate into cells generating the myelin

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sheath [6–8]. Each of the maturation stages displays in addition to the above-described morphological characteristics, a distinct gene expression profile that includes a variety of purinergic receptors, the expression and/or function of which may be developmentally regulated [5, 9–13]. Of the three types of purinergic receptors, metabotropic P1 or adenosine receptors have been functionally implicated in regulating oligodendrocyte progenitor cell migration, proliferation, and differentiation [3, 14–16], while ionotropic P2X receptors have been suggested to induce oligodendrocyte cell death under pathological conditions [3, 14, 17, 18]. Several of the third type of purinergic receptors, namely the G protein-coupled metabotropic P2Y receptors, have been found expressed by cells of the oligodendrocyte lineage [19–21] and functionally described to regulate the migration, proliferation, and differentiation of oligodendrocyte progenitor cells [3, 22, 23]. However, little is currently known about their functional roles for differentiating oligodendrocytes. In this respect, the present study introduces the concept that one of the metabotropic P2Y receptors that has been described as being expressed by differentiating oligodendrocytes and present in myelin, namely the $P2Y_{12}$ receptor, plays a critical role in promoting oligodendrocyte differentiation in part by interacting with an oligodendrocyte secreted protein with extracellular matrix (ECM) properties.

There is increasing evidence that signaling initiated by ECM proteins plays an important role in regulating oligodendrocyte differentiation and CNS myelination [24–29]. Many ECM proteins have been described to promote cell adhesion and to establish a cellular state of strong adhesion and low morphological plasticity. Cells actively undergoing morphological changes such as differentiating oligodendrocytes, however, are thought to require an intermediate state of adhesion that is at least in part mediated through highly controlled signaling events initiated by so-called matricellular ECM proteins [30–33]. Our previous studies identified autotaxin (ATX), also known as ENPP2, phosphodiesterase-Iα/ATX, or lysoPLD, as a protein that is expressed and secreted by differentiating oligodendrocytes and that possesses functional properties of a matricellular ECM protein. More specifically, ATX promotes an intermediate state of adhesion and morphological maturation when differentiating oligodendrocytes are cultured in the presence of fibronectin, an adhesion-promoting and non-permissive ECM substrate for these cells [26, 27, 34–36]. The above-described biological effects of ATX are exerted by its C-terminal domain, here referred to as the modulator of oligodendrocyte remodeling and focal adhesion organization (MORFO) domain, via an active intracellular signaling mechanism. However, the cell surface receptor(s) involved in mediating these biological effects of ATX's MORFO domain remain(s) elusive.

Our previous data provided good evidence for involvement of pertussis toxin-sensitive G proteins, and thus a G protein-coupled receptor (GPCR), in the biological effects mediated by ATX's MORFO domain [35]. Using an in silico approach, we focused on the $P2Y_{12}$ receptor as a good candidate for a GPCR involved in the known biological effects mediated by ATX's MORFO domain for the following reasons. First, the $P2Y_{12}$ receptor has been shown to couple to pertussis toxin-sensitive G proteins in a variety of cell types [37–39]. Second, the $P2Y_{12}$ receptor has functionally been implicated in the modulation of cell adhesion [39–41] and is expressed by oligodendrocytes but not by cells known to be functionally unresponsive to ATX's MORFO domain such as CHO-K1 cells [19, 39, 42]. Third, the primary ligand and agonist characterized for the $P2Y_{12}$ receptor is ADP [37, 43–45]. However, the existence of endogenous surrogate ligands and the formation of function modulating heteromeric receptor–receptor interactions have been proposed for the $P2Y_{12}$ receptor, thus suggesting that yet unknown and more complex signaling mechanisms exist [46, 47]. In this respect, our data reveal a signaling mechanism that is functional in differentiating oligodendrocytes and characterized by the interaction of ATX and in particular its MORFO domain with the $P2Y_{12}$ receptor. More specifically, our data demonstrate that functional responsiveness to ATX's MORFO domain is critically dependent on the presence of the $P2Y_{12}$ receptor, and that the ATX-MORFO-$P2Y_{12}$ signaling axis is crucial for mediating cellular states of intermediate adhesion. Thus, ATX-MORFO-$P2Y_{12}$ signaling appears to play an important role in regulating the morphological/structural plasticity of differentiating oligodendrocytes and, therefore, their ability to efficiently myelinate.

Materials and methods

Animals

Sprague–Dawley female rats with early postnatal litters were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Cell culture

Chinese Hamster Ovary (CHO) cells were cultured under standard conditions (ATCC, Manassas, VA). Primary rat oligodendrocytes were isolated from postnatal rat brains by immunopanning and cultured as described previously [34, 48].

Expression and purification of recombinant protein forms of ATX's MORFO domain

The GST-tagged recombinant proteins rATX-MORFO-GST and rControl-GST were expressed and purified as described previously [34].

For the generation of the human Fc-tagged recombinant form of ATX's MORFO domain, the respective rat sequence was obtained by PCR amplification and inserted into the pFUSE-hIgG4-Fc2 mammalian expression vector (InvivoGen, San Diego, CA) in frame with the amino terminal IL-2 signal sequence and the carboxy terminal human IgG4-Fc domain. Stable transfected COS-7 cell lines were generated and proteins (secreted ATX-MORFO-hIgG4-Fc and hIgG4-Fc alone as control) were purified from serum-free cell culture supernatants using a Protein A mini-column (Bio-Rad, Hercules, CA). Throughout this manuscript, these purified fusion proteins are referred to as rATX-MORFO and rControl. Functionality of the purified rATX-MORFO protein was assessed by morphology assays using primary oligodendrocytes [34]. To mimic an effect on cell morphology equivalent to the use of 50 μg/ml rATX-MORFO-GST, a concentration of 10 μg/ml of rATX-MORFO was found to be sufficient (data not shown). No effect was observed using the control protein at this concentration.

Fluorescence-activated cell sorting

Primary oligodendrocytes were isolated from the brain stems of 2- or 3-day-old rats by immunopanning using A2B5 or O4 antibodies. Immediately after isolation, cells were immunostained using A2B5 (ATCC, Manassas, VA) or O4 hybridoma cell culture supernatants [49–51]. Cells were fixed and permeabilized using the BD Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences, San Diego, CA), incubated first with anti-$P2Y_{12}$ receptor antibodies (Alomone Labs, Jerusalem, Israel) and then with Alexa 488- and Alexa 594-coupled secondary antibodies. Cells were analyzed using a BD FACSCanto II flow cytometer equipped with the BD FACSDiva software package version 6.1.3 (BD Biosciences, San Diego, CA).

Immunocytochemistry

O4 immunostaining of primary oligodendrocytes to be analyzed for morphology was performed as described previously [34]. For double staining of differentiating oligodendrocytes, O4 hybridoma cell culture supernatants and anti-$P2Y_{12}$ receptor antibodies (Alomone Labs, Jerusalem, Israel) were used. To assess the specificity of the immunocytochemical staining, control experiments were performed in which the primary antibodies were omitted. In the case of anti-$P2Y_{12}$ receptor antibodies, control experiments also included pre-incubation with the peptide used for the generation of these antibodies. No staining was observed in any of the control experiments. Labeled cells were analyzed using a Zeiss LSM 510 META NLO laser scanning microscope (Carl Zeiss MicroImaging, Thornwood, NY). Images represent 2D maximum projections of stacks of 0.4 μm optical sections.

siRNA transfection of primary oligodendrocytes and analysis of process morphology

A2B5 immunopanned oligodendrocyte progenitor cells were isolated from postnatal day 2 rat brains and transfected 24 h after initial plating with siRNAs as described previously [24]. The following siRNA duplex oligonucleotides targeting the $P2Y_{12}$ receptor were used: 5′-GUAUCUCGUUCCUCG GAUU-3′ and 5′-AAUCCGAGGAACGAGAUAC (Sigma-Aldrich Co., St. Louis, MO, USA). As control, a pool of non-targeting siRNA duplex oligonucleotides was used (Dharmacon/Thermo Fisher Scientific, Lafayette, CO). Subsequent to siRNA transfection, cells were cultured for 20–24 h under differentiation conditions and then re-plated onto glass coverslips pre-coated with rATX-MORFO-GST or rControl-GST protein (50 μg/ml) mixed with fibronectin (10 μg/ml). Cells were cultured for an additional 48 h, and process network morphology was analyzed as described previously [34]. Briefly, IP Lab imaging software (BD Biosciences Bioimaging, Rockville, MD) was used to determine process index (total area found to be O4-positive after staining minus the cell body) and network area (total area within the radius of the process network surrounding the cell body minus the cell body). For the bar graphs representing process indices and network areas, the mean value for cells cultured on rControl-GST protein was calculated for each of the two parameters in each experiment. This mean value was set to 50 for process index and 100 for network area. Adjusted, i.e., normalized values for all cells were then calculated for each experimental condition.

Generation of stable transfected CHO-K1 cell lines

The $P2Y_{12}$ receptor coding region was amplified by PCR from the mouse-derived I.M.A.G.E. clone 4947057 (ATCC, Manassas, VA) and inserted into the pEF1/V5-His C mammalian expression vector (Invitrogen, Carlsbad, CA). Mouse and rat $P2Y_{12}$ protein sequences are 94% identical. In addition, the pharmacological properties of the murine $P2Y_{12}$ receptor closely resemble those exhibited by the human receptor [52]. Thus, major functional differences between the mouse and rat proteins are unlikely. Stable transfected cell lines expressing V5-His-tagged $P2Y_{12}$ receptor protein (CHO-$P2Y_{12}$ cells) or V5-His-tagged β-galactosidase (CHO-LacZ control cells) were then established using the above expression vector constructs.

Cell spreading and focal adhesion assays

Glass coverslips were pre-coated with rControl or rATX-MORFO protein (10 μg/ml) each in combination with fibronectin (10 μg/ml). CHO-P2Y$_{12}$ and CHO-LacZ cells were pre-incubated after trypsinization in serum-free medium at room temperature with gentle agitation for 1 h prior to plating onto the pre-coated glass coverslips.

To assess cell spreading, cells were allowed to adhere for 1 h and then processed for scanning electron microscopy as described previously [35]. Digital images of spread cells were acquired using a Scanning Electron Microscope (Zeiss EVO 50 XVP; Carl Zeiss MicroImaging, Thornwood, NY). Cell surface areas were measured using the ImageJ software package [53].

To assess focal adhesion organization, cells were either plated directly or after pre-treatment (30 min) with the ROCK inhibitor Y-27632 (10 μM) or a cell-permeable pyrimidine compound that specifically and reversibly inhibits Rac1 (100 μM) [54]. Cells were allowed to adhere for 6 h and the number of paxillin-containing focal adhesions was determined as previously described [34].

Immunoprecipitation

Prior to preparing cell lysates, CHO-P2Y$_{12}$ and CHO-LacZ cells were cultured for 12 h in serum-free medium to eliminate serum-derived ATX. Cells were then washed with ice-cold PBS and lysed for 30 min on ice using IP buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 20% Glycerol, 1% Triton X-100) containing protease/phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail; Thermo Fisher Scientific, Rockford, IL). Cell lysates were cleared by centrifugation (10,000×g for 10 min at 4°C) and incubated overnight at 4°C with anti-V5 or IgG isotype control antibodies (Invitrogen Corp, Carlsbad, CA) and protein G-coupled sepharose beads (GE Healthcare Bio-Sciences Corp; Piscataway, NJ) to immunoprecipitate V5-tagged P2Y$_{12}$ receptor or β-galactosidase. Beads were collected, washed three times with ice-cold IP buffer, and bound proteins were analyzed by Western blotting using anti-ATX antibodies (Cayman Chemical Company, Ann Arbor, MI) as primary antibodies and enhanced chemiluminescence (ECL Plus; Thermo Fisher Scientific Inc., Rockford, IL) for detection.

Results

Oligodendrocytes responsive to the biological effects of ATX's MORFO domain express the P2Y$_{12}$ receptor

As an ideal candidate, GPCR involved in the biological functions of ATX's MORFO domain, the expression of the P2Y$_{12}$ receptor would be expected to correlate in cells of the oligodendrocyte lineage with their ability to respond to ATX's MORFO domain. To test this hypothesis, two populations of cells were analyzed by fluorescence-activated cell sorting (FACS). First, oligodendrocyte progenitor cells that are recognized by the A2B5 antibody and are unresponsive to modulation of cell adhesion by ATX's MORFO domain, and second, differentiating oligodendrocytes that are recognized by the O4 antibody and are responsive to modulation of cell adhesion by ATX's MORFO domain [35]. As shown in Fig. 1a, the intensity of P2Y$_{12}$ receptor staining was found significantly above background in O4-positive differentiating oligodendrocytes, while it appeared similar to background for A2B5-positive oligodendrocyte progenitor cells. This observation is in agreement with previous findings and with the idea that cells functionally responsive to ATX's MORFO domain express significant levels of the P2Y$_{12}$ receptor, while cells that are functionally unresponsive express no or very low levels of the P2Y$_{12}$ receptor [19, 22]. To further confirm the expression of the P2Y$_{12}$ receptor in differentiating oligodendrocytes, primary cultures of oligodendrocytes were double immunostained with O4 and anti-P2Y$_{12}$ receptor antibodies. As shown in Fig. 1b, O4-positive oligodendrocytes are also positive for staining with anti-P2Y$_{12}$ receptor antibodies. No such staining was observed when anti-P2Y$_{12}$ receptor antibodies were pre-incubated with the peptide used for the generation of these antibodies (data not shown) or when oligodendrocyte progenitor cells were double immunostained with A2B5 and anti-P2Y$_{12}$ receptor antibodies (Fig. 1c).

siRNA-mediated knockdown of P2Y$_{12}$ receptor expression attenuates the effects of ATX's MORFO domain on the process morphology of differentiating oligodendrocytes

We have shown previously that ATX's MORFO domain promotes the establishment of a complex and expanded process network by differentiating oligodendrocytes [34]. To assess the role of the P2Y$_{12}$ receptor in this functional effect of ATX's MORFO domain, P2Y$_{12}$ receptor expression was knocked down using siRNA-mediated gene silencing. At the time of analysis, protein levels of the P2Y$_{12}$ receptor were found to be significantly reduced compared to control conditions (Supplementary Fig. S1). In addition, process index and network area (for definitions see "Materials and methods") were found to not be significantly different upon knock-down of P2Y$_{12}$ receptor expression under the conditions used here (Fig. 2; compare siControl-rControl-GST with siP2Y$_{12}$-rControl-GST; p=0.502 and 0.374 for process index and network area, respectively). Thus, P2Y$_{12}$ receptor expression appears insufficient to significantly modulate the parameters of cell morphology tested at the developmental

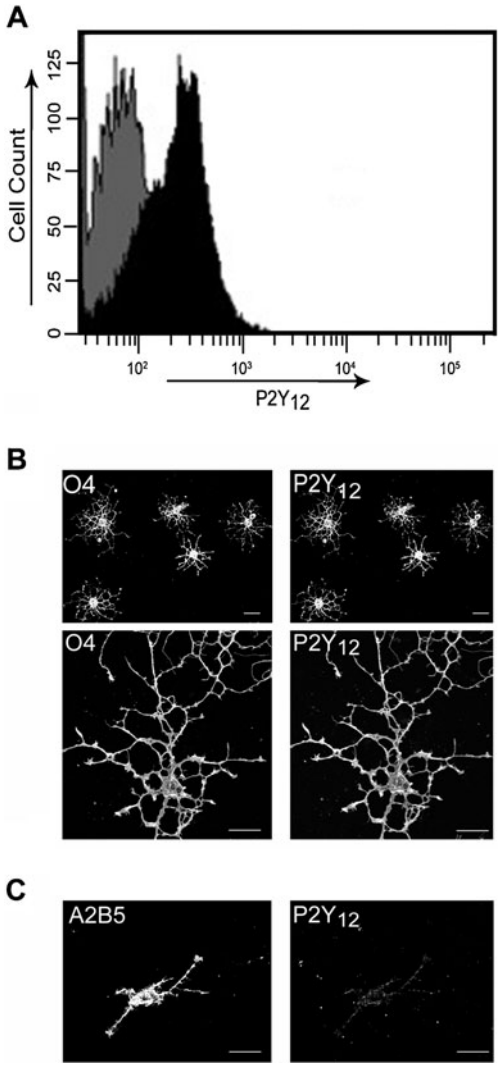

Fig. 1 The $P2Y_{12}$ receptor is expressed by differentiating oligodendrocytes. **a** Representative FACS histogram overlay of A2B5 immunopanned oligodendrocyte progenitor cells isolated from postnatal day 2 rat brainstems (gray histogram) and O4 immunopanned differentiating oligodendrocytes isolated from postnatal day 3 brainstems (*black histogram*) both stained using anti-$P2Y_{12}$ antibodies. The intensity of staining is depicted on the *x*-axis. The histogram of cells stained with isotype control antibodies overlaps with the gray histogram (not shown). **b** Immunostaining of primary oligodendrocytes in culture using O4 (*left*) and anti-$P2Y_{12}$ antibodies (*right*). Differentiating oligodendrocytes were derived from oligodendrocyte progenitor cells isolated by A2B5 immunopanning from P2 rat brains, cultured for 24 h under proliferation conditions and then for 3 days under differentiation conditions. *Scale bars*, 20 μm. **c** Immunostaining of primary oligodendrocyte progenitor cells in culture using A2B5 (*left*) and anti-$P2Y_{12}$ antibodies (*right*). Oligodendrocyte progenitor cells were derived from A2B5 immunopanned cells (P2 rat brains), cultured for 48 h under proliferation conditions. *Scale bars*, 20 μm

time-point assessed. It is of note that this developmental time-point refers to stages of the oligodendrocyte lineage, at which endogenous ATX expression is relatively low, but cells are functionally responsive to the addition of exogenous ATX [34, 35]. Thus, and consistent with our previous findings [34], addition of ATX's MORFO domain led to a significant increase in process index and network area (Fig. 2, siControl). In contrast, when cells were transfected with the $P2Y_{12}$ receptor-specific siRNA, no significant change in process index or network area was noted upon addition of ATX's MORFO domain (Fig. 2, siP2Y12). These data demonstrate that $P2Y_{12}$ receptor expression is necessary for ATX's MORFO domain to exert its effects on the morphology of differentiating oligodendrocytes.

Exogenous expression of the $P2Y_{12}$ receptor renders CHO-K1 cells functionally responsive to ATX's MORFO domain

To assess the extent to which $P2Y_{12}$ receptor expression may be a critical determinant of overall functional responsiveness to ATX's MORFO domain, we used CHO-K1 cells as a model system. CHO-K1 cells do not endogenously express the $P2Y_{12}$ receptor, and they are unresponsive with regard to known effects of ATX's MORFO domain (Figs. 3, 4 and [55, 56]). They do, however, express fibronectin receptors, thus allowing the use of fibronectin as an adhesion-promoting substrate and keeping with the experimental paradigms used in our previous studies [34, 57]. To assess the extent to which $P2Y_{12}$ receptor expression may render CHO-K1 cells responsive to ATX's MORFO domain, a cell line stably expressing the $P2Y_{12}$ receptor was generated (CHO-$P2Y_{12}$ cells). As control, a cell line expressing β-galactosidase (CHO-LacZ cells) was generated in parallel. To evaluate the functionality of the $P2Y_{12}$ receptor expressed in CHO-$P2Y_{12}$ cells, we performed two functional assays. First, actin stress fiber formation in response to 2-methylthioadenosine diphosphate (2-MeS-ADP), a non-hydrolyzable analog of ADP, was assessed. It has been previously demonstrated [58] that 2-MeS-ADP induces the assembly of actin stress fibers in $P2Y_{12}$ receptor-expressing but not control CHO cells. As shown in Supplementary Fig. S2A and consistent with the previous data, the presence of stress fibers was found to be increased in CHO-$P2Y_{12}$ but not CHO-LacZ cells upon treatment with 2-MeS-ADP. Second, inhibition of forskolin-dependent cAMP accumulation upon treatment with 2-MeS-ADP was determined. Previous studies demonstrated that ADP modulates stimulation-dependent, but not spontaneous, cAMP accumulation [39]. In particular, it was shown that ADP via activation of the $P2Y_{12}$ receptor inhibits stimulation-dependent cAMP accumulation [39, 45]. As shown in Supplementary Fig. S2B, forskolin-stimulated cAMP accumulation was inhibited in CHO-$P2Y_{12}$ but not CHO-LacZ cells upon treatment with 1 μM 2-MeS-ADP. Consistent with a previously reported EC_{50} of 60 nM [45], a statistically significant effect was not observed at a concentration of 10 nM 2-MeS-ADP. Together, these data demon-

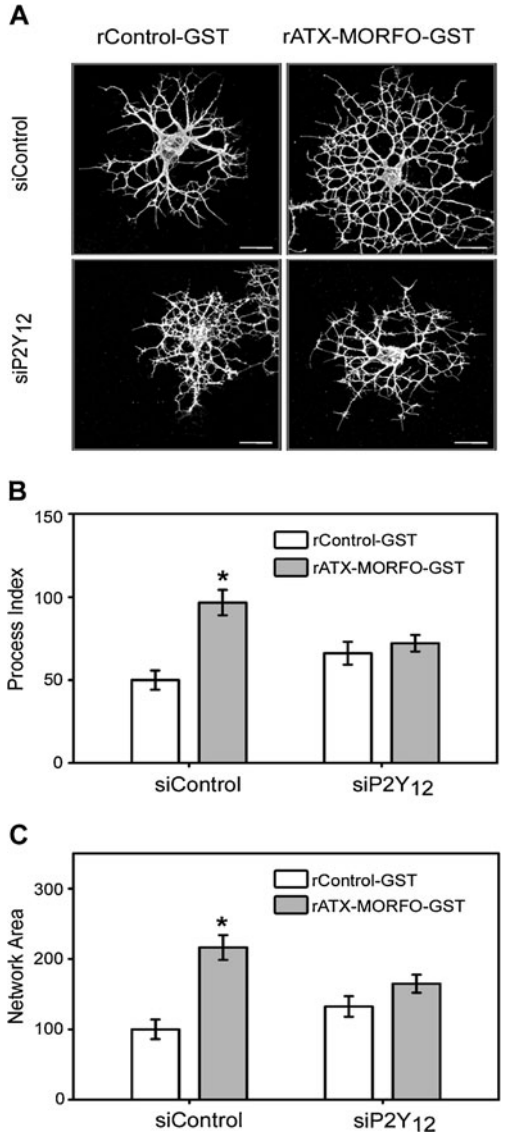

Fig. 2 siRNA-mediated knockdown of $P2Y_{12}$ receptor expression attenuates the effects of ATX's MORFO domain on the process morphology of differentiating oligodendrocytes. Oligodendrocyte progenitor cells were isolated by A2B5 immunopanning from postnatal day 2 rat brains and treated with either an siRNA against the $P2Y_{12}$ receptor ($siP2Y_{12}$) or a control siRNA pool (siControl). Cells were then allowed to differentiate in the presence of a recombinant form of ATX's MORFO domain (rATX-MORFO-GST) or control protein (rControl-GST) and analyzed 72 h after siRNA transfection. **a** Representative images of cells stained with O4 antibodies. *Scale bars*, 20 μm. **b**, **c** Bar graphs representing quantitative analyses of process index (**b**) and network area (**c**). The mean values for cells treated with control siRNA (siControl) and control protein (rControl-GST) were set to 50 and 100 for process index and network area, respectively. All values were then calculated accordingly. In all bar graphs, mean values and SEMs of at least three independent experiments are shown. At least 30 cells per condition and experiment were analyzed in three independent experiments, that is a total of at least 90 cells per condition. *Stars* indicate an overall two-tailed significance level of $p<0.05$ as determined by Student's *t* test analysis

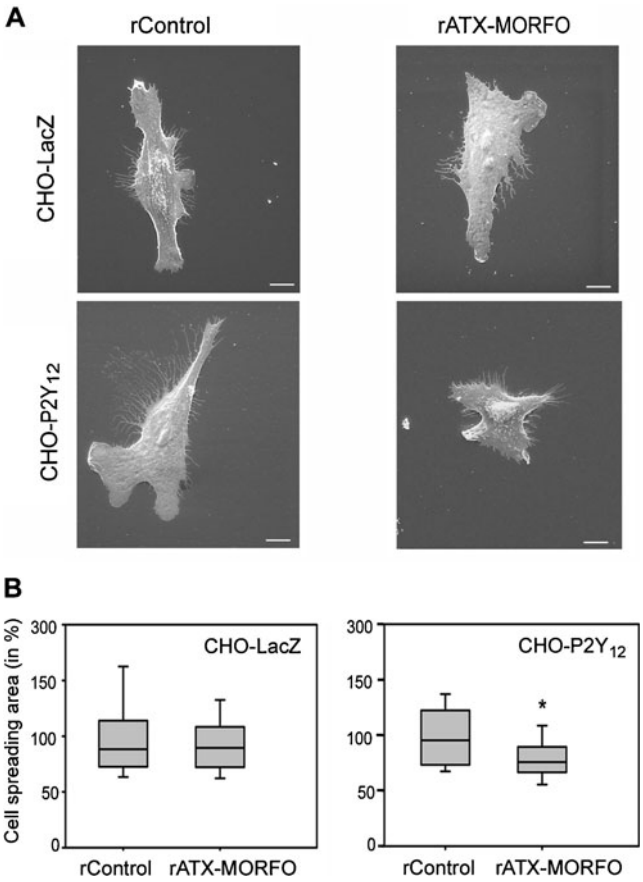

Fig. 3 $P2Y_{12}$ receptor expression renders CHO-K1 cells responsive to morphological changes mediated by ATX's MORFO domain. A CHO-K1-derived cell line stably expressing the $P2Y_{12}$ receptor ($CHO\text{-}P2Y_{12}$) was assessed for changes in cell spreading in response to ATX's MORFO domain (rATX-MORO). As controls, a cell line expressing β-galactosidase (CHO-LacZ) and control recombinant protein (rControl) were used. **a** Representative scanning electron micrographs of $CHO\text{-}P2Y_{12}$ and CHO-LacZ cells plated on rControl- or rATX-MORO-containing substrate. *Scale bars*, 10 μm. **b** Box and whisker plots depicting the cell spreading area of $CHO\text{-}P2Y_{12}$ and CHO-LacZ cells plated on rATX-MORO- or rControl-containing substrate. The mean value for cells plated on rControl-containing substrate was set to 100% and values for all cells were calculated accordingly. The plots depict medians and quartiles of three independent experiments. At least 20 cells per condition and experiment were analyzed, that is at least 60 cells per condition. Whiskers represent the 10th and 90th percentile. The *star* indicates an overall significance level of $p<0.05$ as determined by the Mann–Whitney Rank Sum test

strate that the $CHO\text{-}P2Y_{12}$ cell line generated by us expresses a functionally active $P2Y_{12}$ receptor.

Our previous findings suggested that ATX's MORFO domain mediates cellular states of intermediate adhesion [35] in which cell spreading is reduced compared to states of strong adhesion in which cell spreading is a predominant feature [31]. Thus, to assess the effects of ATX's MORFO domain on cell morphology, CHO-LacZ and $CHO\text{-}P2Y_{12}$ cells were cultured in the presence of ATX's MORFO domain or control protein, and the area occupied by each

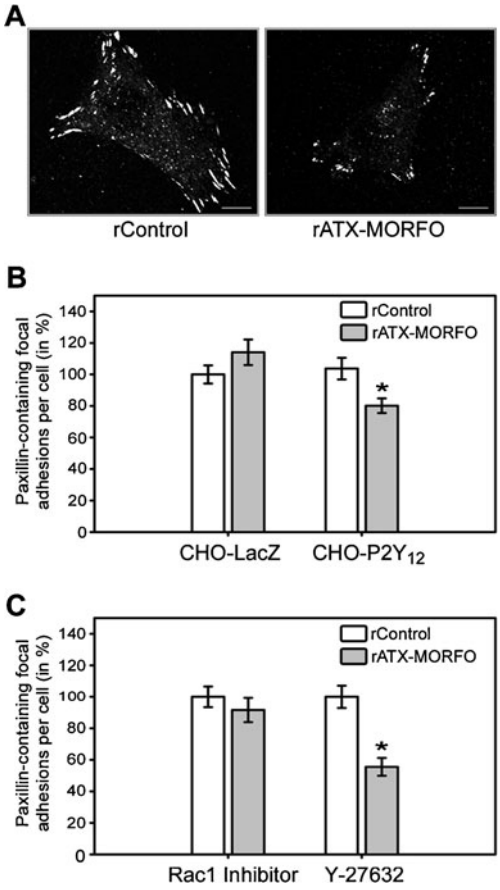

Fig. 4 $P2Y_{12}$ receptor expression renders CHO-K1 cells responsive to changes in focal adhesion organization mediated by ATX's MORFO domain. A CHO-K1-derived cell line stably expressing the $P2Y_{12}$ receptor (CHO-$P2Y_{12}$) was assessed for changes in the number of paxillin-containing focal adhesions per cell in response to ATX's MORFO domain (rATX-MORO). As controls, a cell line expressing β-galactosidase (CHO-LacZ) and control recombinant protein (rControl) were used. **a** Representative images of CHO-$P2Y_{12}$ cells plated on rControl- or rATX-MORFO-containing substrate and immunostained for paxillin. Confocal images represent optical sections approximately 250 nm in depth and close to the basal surface. Scale bars, 20 μm. **b**, **c** Bar graphs depicting the number of paxillin-containing focal adhesions per cell as percent of control (mean of control=100%) in untreated cells (**b**) and in cells pre-treated with a Rac1 inhibitor or the ROCK inhibitor Y-27632 (**c**). The mean values for cells plated on rControl-containing substrate were set to 100% and all values were calculated accordingly. Mean values and SEMs of at least three independent experiments are shown. At least 20 cells per condition and experiment were analyzed, that is at least 60 cells per condition. *Stars* indicate an overall two-tailed significance level of $p<0.05$ as determined by Student's *t* test analysis

cell was determined using scanning electron microscopy in combination with the area measurement tool of Image J (Fig. 3). No significant differences in the areas occupied by CHO-LacZ cells were noted when comparing the addition of ATX's MORFO domain to the addition of control protein. In contrast, when culturing CHO-$P2Y_{12}$ cells in the presence of ATX's MORFO domain, the spreading areas were found significantly reduced compared to the spreading areas of CHO-$P2Y_{12}$ cells cultured in the presence of control protein. These data confirm a functional role of ATX's MORFO domain in mediating intermediate states of adhesion. Most importantly, they demonstrate that $P2Y_{12}$ receptor expression is critical for ATX's MORFO domain to exert its functional effects on the cell morphology of CHO cells in a way that is similar to what has been observed for primary oligodendrocytes.

Intermediate states of adhesion as mediated by ATX's MORFO domain are associated with a reorganized assembly of multimolecular protein complexes mediating a link between the ECM and the cytoskeleton that are called focal adhesions [31, 59, 60]. Accordingly, our previous data demonstrated that the number of paxillin-containing focal adhesions was reduced in oligodendroglial cells in the presence of ATX's MORFO domain when compared to control protein [34]. In light of the tight functional association between focal adhesion organization and cell morphology, a requirement for $P2Y_{12}$ expression for the effects of ATX's MORFO domain on focal adhesion organization was anticipated. Indeed, treatment of CHO-$P2Y_{12}$ cells with ATX's MORFO domain significantly reduced the number of paxillin-containing focal adhesions when compared to treatment with control protein (Fig. 4). In contrast, no change in the number of paxillin-containing focal adhesions was noted when comparing CHO-LacZ cells treated with ATX's MORFO domain to those treated with control protein. These results demonstrate that $P2Y_{12}$ receptor expression is necessary for the effects of ATX's MORFO domain on focal adhesion organization.

Main regulators determining adhesive states are members of the Rho family of small GTPases, whereby prolonged activation of Rac, but not Rho, has been associated with the establishment of intermediate states of adhesion [31]. Thus, we assessed the involvement of Rho and Rac in the effects on focal adhesion organization mediated by ATX's MORFO domain and requiring $P2Y_{12}$ expression. As shown in Fig. 4c, pre-treatment with the Rac1-inhibitor, but not the Rho-kinase inhibitor, was able to inhibit the effects of ATX's MORFO domain on focal adhesion organization in CHO-$P2Y_{12}$ cells.

ATX interacts with the $P2Y_{12}$ receptor

The above demonstrated necessity and requirement of the $P2Y_{12}$ receptor for the effects mediated by ATX's MORFO domain revealed the existence of a functional interaction between these two molecules. To determine the extent to which this interaction involves complex formation between ATX and the $P2Y_{12}$ receptor, immunoprecipitation experiments were performed. V5-tagged $P2Y_{12}$ receptor and β-galactosidase were immunoprecipitated from CHO-$P2Y_{12}$ and CHO-LacZ cells, respectively. As shown in Fig 5a, ATX was detected in the immunoprecipitation fraction of CHO-$P2Y_{12}$, but not CHO-LacZ cells. Both cell lines were found to express comparable levels of endogenous ATX (data

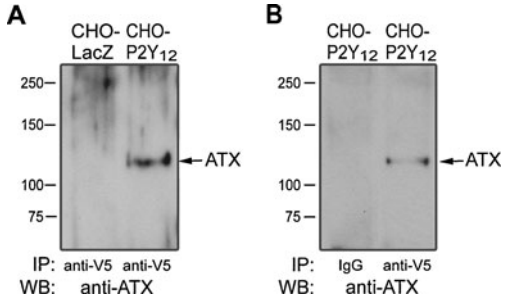

Fig. 5 ATX interacts with the $P2Y_{12}$ receptor. Western blot (WB) analysis of ATX on immunoprecipitated (IP) protein fractions. **a** Lysates prepared from CHO-$P2Y_{12}$ and CHO-LacZ cells were used for immunoprecipitation with anti-V5 antibodies recognizing the V5-tagged $P2Y_{12}$ receptor (in CHO-$P2Y_{12}$ cells) or β-galactosidase (CHO-LacZ cells). **b** Lysates prepared from CHO-$P2Y_{12}$ were used for immunoprecipitation with anti-V5 or isotype control (IgG) antibodies. Numbers to the left **(a, b)** indicate molecular weight markers in kilodalton

not shown). In addition, no ATX protein was detected in the immunoprecipitation fraction of CHO-$P2Y_{12}$ cells when isotype control antibodies were used to obtain the immunoprecipitation fraction (Fig. 5b). Taken together, these data demonstrate that ATX and the $P2Y_{12}$ receptor can physically interact with each other.

Discussion

Our data presented here demonstrate that $P2Y_{12}$ receptor expression is a critical determinant of functional responsiveness to ATX's MORFO domain. More specifically, promotion of the morphological maturation of differentiating oligodendrocytes by ATX's MORFO domain was found to be critically dependent on the presence of the $P2Y_{12}$ receptor. Furthermore, exogenous expression of the $P2Y_{12}$ receptor in cells otherwise unresponsive to ATX's MORFO domain was found to render these cells responsive to the effects of ATX's MORFO domain on cell morphology and focal adhesion organization. The ATX-$P2Y_{12}$ receptor-mediated effect on focal adhesion organization was found to involve the activation of Rac1, but not Rho. Lastly, our data demonstrate that ATX and the $P2Y_{12}$ receptor can interact with each other not only functionally but also physically. Thus, our data reveal a signaling mechanism that is characterized by a functional and physical interaction between ATX and the $P2Y_{12}$ receptor and that appears crucial for mediating cellular states of intermediate adhesion and, therefore, morphological/structural plasticity.

While our data demonstrate a physical interaction between ATX and the $P2Y_{12}$ receptor, the exact molecular mechanism underlying this interaction is currently unclear. In a simple model, ATX via its MORFO domain would directly bind to the $P2Y_{12}$ receptor and activate a signaling pathway leading to cellular states of intermediate adhesion and morphological/structural plasticity. In addition, however, heteromeric receptor complex formation and receptor cross talk have been reported and/or suggested to modulate signaling involving a number of P2Y receptors including the $P2Y_{12}$ receptor [46, 61]. Additional studies will be necessary to determine the extent to which such receptor complex formation and/or cross talk may be involved in the ATX-MORFO-$P2Y_{12}$ interaction described here.

Cellular states of strong adhesion and low morphological plasticity are typically mediated by ECM proteins interacting with receptors of the integrin family. Conferring an intermediate state of adhesion in the presence of an otherwise adhesion-promoting ECM protein as demonstrated for ATX via its MORFO domain, could, therefore, be achieved by blocking integrin binding to the adhesive ECM protein. In this regard, it is of note that ATX has been recently described to directly interact with cell surface integrins [62, 63]. This interaction is, however, mediated through ATX's N-terminal somatomedin B-like domains, which are not present in the recombinant forms of ATX's MORFO domain used in these and our earlier studies. In addition, our data demonstrate a clear requirement for intracellular signaling through pertussis toxin-sensitive G proteins [35, 36]. Thus, we consider it unlikely that direct ATX–integrin interactions contribute to the biological effects of ATX's MORFO domain described here.

Our data demonstrate that an interaction between ATX and the $P2Y_{12}$ receptor regulates oligodendrocyte differentiation. It is of note that $P2Y_{12}$ receptor immunoreactivity has been recently described to be inversely proportional to demyelination and lesion formation in one form of the human demyelinating disease multiple sclerosis (MS) [67]. Thus, a lack of $P2Y_{12}$ receptor signaling may be one of the mechanisms underlying inefficient repair of the myelin sheath within MS lesions, thereby contributing to pathology in MS.

Our data are in support of an ATX-MORFO-$P2Y_{12}$ signaling mechanism(s) regulating oligodendrocyte differentiation. However, ADP is the primary ligand and agonist characterized for the $P2Y_{12}$ receptor. In this regard, it remains unknown to what extent an ADP-initiated $P2Y_{12}$ receptor signaling pathway may regulate oligodendrocyte differentiation. Purinergic signaling is known to control oligodendrocyte differentiation and myelination [4, 5]. However, a role of ADP-$P2Y_{12}$ signaling has to our knowledge not yet been investigated. Based on the known data on $P2Y_{12}$ receptor signaling, it is plausible that ADP-$P2Y_{12}$ signaling may exert an adhesion-promoting and thus maturation restricting effect on differentiating oligodendrocytes. Thus, an equilibrium may exist between adhesion-promoting ADP-$P2Y_{12}$ and adhesion-modulating ATX-MORFO-$P2Y_{12}$ signaling. Adhesion-modulating ATX-MORFO-$P2Y_{12}$ signaling has been found here to involve Rac1 activity, whereas adhesion-promoting ADP-$P2Y_{12}$ signaling is thought to involve Rho activity [39].

Interestingly, purine nucleotides and nucleosides including ADP are released into the extracellular space following CNS injury, and inhibitory effects on the differentiation of remyelinating oligodendrocytes can be overcome by inhibiting Rho activity [64–66]. Thus, it is tempting to speculate that a well-balanced homeostasis of ADP-$P2Y_{12}$-Rho and ATX-MORFO-$P2Y_{12}$-Rac signaling regulates oligodendrocyte differentiation and myelination. Any dysregulation of this homeostasis by for example an excess of ADP-$P2Y_{12}$-Rho signaling upon injury could then contribute to pathology. In conclusion, by revealing an interaction between ATX and the $P2Y_{12}$ receptor, we have gained a better understanding of the molecular mechanisms that regulate oligodendrocyte differentiation during development and that may be involved in limiting myelin repair under pathological conditions.

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