Mimicking extracellular matrix-mediated mechano-activation by antibodies to control signaling of the adhesion G protein-coupled receptor GPR126/ADGRG6

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Abstract

The adhesion G protein-coupled receptor (aGPCR) GPR126/ADGRG6 plays an important role in several physiological functions, such as myelination, peripheral nerve repair and osteoblast differentiation, which renders the receptor an attractive pharmacological target. GPR126 is a mechano-sensor that incorporates signals from the extracellular matrix (ECM) through binding to its N-terminal ligands collagen IV and laminin 211. Since ECM components are not suitable therapeutics alternate compounds with more favorable characteristics would be desirable that can mimic the physiologic activation pattern. Antibodies could present an apt alternative as they can specifically target the N-terminus of the receptor, reach multiple tissue compartments and have been shown to modulate GPCR activity levels.

In this study, we use a monoclonal antibody targeting an N-terminal HA-epitope to induce receptor signaling, which can be enhanced through the addition of secondary antibodies. Using single cell atomic force microscopy (AFM) in combination with a fluorescent cAMP sensor, we show that antibody-mediated activation is achieved through combined pushing and pulling forces, while collagen IV and laminin 211 only mediate GPR126 activation through pushing or pulling, respectively. Thus, the antibody-mediated approach can reliably mimic the activation induced by both endogenous ligands. Our findings show that antibody-mediated activation for GPR126 is feasible and can be used for precise targeting of this receptor, thereby establishing it as a pharmaceutical target.

Introduction

The adhesion G protein coupled receptor (aGPCR) GPR126/ADGRG6 plays an essential role in several important physiologic and pathogenic processes, including myelination (*Mogha et al., 2013; Monk et al., 2009; Monk et al., 2011; Ravenscroft et al., 2015*), peripheral nerve injury and repair (*Mogha et al., 2016*), the development of the peripheral nervous system (PNS) (*Ravenscroft et al., 2015*), and the differentiation of osteoblasts (*Sun et al., 2020*) and adipocytes (*Suchý et al., 2020*). Further, an association of *GPR126* variants with the development of scoliosis was found in humans and mice (*Karner et al., 2015; Kou et al., 2013; Kou et al., 2018; Liu et al., 2018; Man et al., 2019; Qin et al., 2017; Takeda et al., 2019; Xu et al., 2015; Xu, E. et al., 2019b; Xu, E. et al., 2019a; Xu, L. et al., 2019*). Thus, pharmacological targeting of this receptor is of high interest. The physiological implications are mainly attributed to the modulation of cAMP levels by the receptor, which is achieved through the receptor's coupling to G_s protein (*Mogha et al., 2013*). The N-terminus of GPR126 includes 5 structurally different domains (*Leon et al., 2020*) among them the autoproteolysis-inducing (GAIN) domain (*Arac et al., 2012*) at which the receptor is autoproteolytically cleaved at the conserved HLT motif within the GPCR proteolytic site (GPS) (*Lin et al., 2004; Moriguchi et al., 2004*). Further, GPR126 undergoes furin-mediated cleavage within the SEA domain (*Leon et al., 2020*).

Like other aGPCRs GPR126 harbors an endogenous tethered agonistic sequence located distal of the GPS cleavage motif, termed the *Stachel* sequence (*Liebscher et al., 2014*). Synthetic peptides derived from this integral agonist can be used as agonists on the receptor (*Demberg et al., 2017; Liebscher et al., 2014*). Further, small molecule agonists have been identified (*Bradley et al., 2019; Diamantopoulou et al., 2019*), but similar to the agonistic peptides, they lack specificity for the receptor (*Demberg et al., 2017*). Additional activation can be achieved through the receptor's ligands, which each bind to specific parts of the N-terminus. Collagen IV (*Paavola et al., 2014*) and Prion protein PrP^C (*Küffer et al., 2016*) directly activate the receptor to increase cAMP levels. Laminin 211 (*Petersen et al., 2015*), however, has a more complex mode of action. Under static conditions laminin 211 inhibits cAMP formation, but it enhances cAMP levels after addition of mechanical forces. Besides their different modes of action, both laminin 211 and collagen IV are components of the basal lamina, where they play an important role in GPR126-mediated myelination. This indicates that these extracellular matrix (ECM) ligands modulate the activity of GPR126 in distinct and specific manners.

ECM proteins do not display the characteristics of a classic receptor agonist as they are long-lived, stable molecules with essentially no diffusivity. Thus, it is unclear how the interaction

between the aGPCR and the ECM can be interpreted as a signal to modulate receptor activity levels. Mechanical forces generated by the interaction of the aGPCR with the ECM are theorized to represent this signal (*Liebscher et al., 2014; Stoveken et al., 2015*). This theory is supported by the observed second messenger generation upon shaking and vibration of cells overexpressing GPR126 (*Petersen et al., 2015*) and another aGPCR GPR114 (*Wilde et al., 2016*). However, the force input needed to activate these aGPCRs has not been defined. Major obstacles in studying ligand-induced mechano-activation of aGPCRs are the unfavorable physicochemical characteristics of ECM interaction partners, such as poor solubility, as well as the low specificity of the interaction between the ECM and the receptor. An additional difficulty stems from the non-physiological properties of experimental models of mechano-activation, such as vibration and shaking. Such models not only fail to quantify the magnitude of forces needed for receptor activation, but are also not amenable to pharmacological screens. Being able to mimic the ECM-mediated activation patterns on GPR126 will significantly increase our understanding of these functional interactions and structure-function relations in mechano-transduction.

In this study, we use an antibody recognizing an N-terminal HA epitope on GPR126 as an agonist, whose activity-inducing effects is modulated through incubation with a secondary antibody. We employ this setup to investigate the roles of the N-terminus and cleavage sites of GPR126 for mechano-activation. Using single cell atomic force microscopy (AFM) in combination with a fluorescent cAMP sensor, we determine in real time the type and strength of mechanical input needed to activate GPR126 through the antibody-based approach, and through the natural GPR126 ligands collagen IV and laminin 211. We show that the antibody mediates mechanical forces that are also employed by the ligands, which allows to mimic the physiological activation patterns of these complex proteins using a specific, easy to modify molecule. Our findings have mechanistic implications for the activation mechanisms of GPR126 and likely other aGPCRs and might set the stage for pharmaceutical targeting of the receptor.

Results

An antibody targeting the N-terminal HA epitope can activate GPR126

Since GPR126 can be activated through interaction with its ligands and mechanical stimuli, it can be assumed that the N-terminus plays a decisive role in mediating these signals. However, our understanding of these dynamic processes is limited. In order to mimic mechanical forces that could activate GPR126, we established an antibody-based approach, since antibodies show high-affinity and highly specific interactions with their antigens. Because there is no antibody available targeting GPR126 directly at the N-terminus, we used the hemagglutinin (HA) epitope for our experimental setup and inserted it right after the predicted signal peptide of wildtype (WT) GPR126 (Fig. 1A). Increasing concentrations of a commercially available anti-HA antibody significantly elevated cAMP levels in COS-7 cells transfected with the HA-tagged GPR126 (Fig. 1B), but not in the empty vector transfected control cells.

Adding increasing concentrations of a secondary antibody to a stable concentration of 1 µg/ml of the anti-HA antibody yielded an even stronger activation of the receptor (Fig. 1C/D). In order to elucidate whether the observed activation is a consequence of receptor cross-linking through the antibodies or due to the additional weight being attached to the receptor N-terminus, we added anti-HA antibody-conjugated paramagnetic Dynabeads®, which are decisively larger in size than antibodies, to cells transfected with HA-tagged GPR126 (Fig. 1E). We observed a significant increase in cAMP levels (Fig. 1F), which was comparable with anti HA-mediated activation alone (Fig. 1B) but lower than the combination of primary and secondary antibody (Fig. 1E), indicating that simply adding weight is not the sole key to GPR126 activation. In line with this observation, exposure to a 700 Gs magnetic field placed below the cell layer cannot further enhance conjugated Dynabead-mediated activation (Suppl. Fig. S1A). In a control experiment with an anti-FLAG antibody targeting the C-terminal epitope of GPR126 no change in cAMP levels was observed (Supp. Fig. S1B).

Figure 1. Antibodies against the N-terminus activate GPR126. (A) Schematic setup for the anti-HA antibody (anti-HA ab) stimulation of full-length WT GPR126 in cAMP accumulation assays (B). Different concentrations of anti-HA antibody were used to treat vector control (pcDps) and GPR126-transfected COS-7 cells (effect of construct $p = 0.0410$, effect of concentration $p < 0.0001$, interaction construct $\times$ concentration $p = 0.0506$; two-way ANOVA). Basal cAMP level in pcDps transfected cells: 8.9 ± 0.8 nM/well. (C, D) Amplification of cAMP signal of GPR126 with 1 µg/ml of anti-HA ab and subsequent incubation with different concentrations of secondary ab on vector control and GPR126-transfected COS-7 cells (effect of construct $p = 0.0104$, effect of

concentration $p < 0.0001$, interaction construct $\times$ concentration $p = 0.0072$; two-way ANOVA). Basal cAMP level in pcDps transfected cells: 4.2 ± 1.0 nM/well. (E, F) Paramagnetic Dynabeads® were conjugated with anti-HA ab and used in different concentrations for stimulation of vector control and GPR126-transfected COS-7 cells (effect of construct $p = 0.0001$, effect of concentration < 0.0001 , interaction construct $\times$ concentration $p < 0.0001$; two-way ANOVA). Empty vector (pcDps) served as negative control. Basal cAMP level in pcDps transfected cells: 5.8 ± 0.4 nM/well. All data are given as means $\pm$ SEM of three – five independent experiments each performed in triplicates. Statistics were performed by applying a 2-way ANOVA followed by Dunnett post hoc analysis; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All significances given above individual points in the graphs show the result of the post hoc analysis. Schematic images were created with BioRender.com.

Anti-HA antibody-mediated activation depends on the *Stachel* sequence as well as GPS cleavage and is obliterated upon N-terminal prolongation

The N-terminus of GPR126 contains several functional domains, which can be subject to tissue-specific splicing (*Knierim et al., 2019*) and serve to interact with the known ligands collagen IV (*Paavola et al., 2014*), laminin 211 (*Petersen et al., 2015*) and prion protein (*Küffer et al., 2016*). To investigate their role in mechano-sensing, we used different N-terminal deletion mutants of GPR126, whose basal activity and expression levels were previously reported (*Petersen et al., 2015*). The constructs generated were: Δ CUB (lacking 109 aa compared to the WT), Δ PTX (lacking 206 aa) and Δ CUB Δ PTX (lacking 315 aa). Additionally, we generated an N-terminally prolonged variant of GPR126 containing an mRuby-tag (236 aa prolongation compared to the WT) (Fig. 2A). Comparable protein expression levels of the mutants were demonstrated with cell surface and total ELISA assays in order to detect cell membrane and total protein expression, respectively (Suppl. Fig. S2A). The capability of receptor activation for each mutant was measured in cAMP accumulation assays using the synthetic GPR126 *Stachel* peptide as stimulus, thereby ensuring undisturbed signaling (Suppl. Fig. S2B). We stimulated these mutants with the same antibody-based setup as described in Fig. 1 and found that deletion of the CUB and PTX domains yields results highly similar to WT GPR126 (Fig. 2B). The N-terminally prolonged mRuby construct, despite showing WT-like expression and peptide activation (Suppl. Fig. S2A/B), could not be activated through antibodies (Fig. 2B).

The complex architecture of the N-terminus of GPR126 includes two cleavage sites; the GPCR proteolytic site (GPS) within the GAIN domain, and a furin site, located in the SEA domain (Fig. 2C). To analyze whether cleavage at either position may be required for antibody-mediated activation, we stimulated the furin-cleavage-deficient receptor mutant R468A, the GPS cleavage mutant H839R, and the double mutant R468A H838R with the same paradigm

(Fig. 2D). Prior to that, proper protein expression was confirmed with cell surface and total ELISA (Suppl. Fig. S2A) and receptor activation via *Stachel* peptide stimulation was demonstrated (Suppl. Fig. S2B). Proof of effective cleavage-deficiency was shown in Western Blot with anti-HA antibody (Suppl. Fig. S2C). Interestingly, only the furin mutant R468A retained increased cAMP levels upon antibody incubation, while no activation was seen for the other mutants (Fig. 2D). These results suggest that furin cleavage is neglectable for antibody-based mechano-activation, whereas GPS cleavage seems to be required. Similarly, mutation of the *Stachel* sequence (F843A, Fig. 2C) obliterated antibody-induced activation of GPR126 (Fig. 2D), proving that the antibody does not directly activate the receptor but has to engage the encrypted agonistic sequence.

Figure 2. Antibody-mediated activation of GPR126 depends on autoproteolytic cleavage and is obliterated in a construct with an elongated N-terminus. (A) The domain architecture of the human GPR126 WT and respective mutants is depicted. The N-terminus contains the signal peptide (SP, orange triangle), a Complement C1r/C1s-Uegf-BMP1 domain (CUB, magenta oval), a pentraxin domain (PTX, purple square) and the Sperm protein, Enterokinase and Agrin (SEA) domain (blue hexagon) including the furin site. The highly conserved GAIN domain (green rectangle) contains the GPS, which is followed by the *Stachel* sequence (S). In our receptor constructs, we inserted a N-terminal hemagglutinin tag (HA-TAG, red pentagon) immediately distal to the signal

peptide a and a C-terminal Flag tag (FLAG-TAG, yellow pentagon) right before the Stop codon. **(B)** The receptor variants were transfected into COS-7 cells and analyzed with the same antibody-based approach as used in Fig. 1. **(C, D)** The furin-deficient receptor mutant R468A, the GPS cleavage mutant H839R, double mutant R468A H838R, and the *Stachel* mutant F843A are displayed **(C)** and tested in cAMP assays **(D)**. Images were created with BioRender.com. Empty vector (pcDps) served as negative control (cAMP level: 4.3 ± 0.7 nM/well). Data are given as mean $\pm$ SEM of three – eight different experiments each performed in triplicates. Statistics were performed by applying a 1-way ANOVA followed by Dunnett post hoc analysis; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All shown statistical significances compare the condition to the basal cAMP accumulation of the same construct.

Anti HA antibody activates GPR126 through pushing and pulling forces

In order to specify the mechanical forces needed to activate GPR126 through the anti-HA antibody, we employed an atomic force microscopy (AFM) approach. We theorized that the antibody could exert either pulling (through cross-linking) or pushing forces (through residing on the cell layer) on the receptor, and that both can be quantified with AFM. To do so, tipless AFM cantilevers were coated with anti-HA antibody using PEG linkers according to a well-established protocol (*Ebner et al., 2007*). In contrast to cantilevers with a tip, this approach allows for multiple antibodies to bind and thus interact with the cell surface. Therefore, the mechanical force induced by the cantilever and mediated by the antibodies is applied to multiple receptors expressed on the cell surface simultaneously. The coated cantilevers were then used to apply pressure (pushing) or a force-clamp (pulling) to individual cells successfully co-transfected with the given receptor construct and the Pink Flamindo cAMP sensor (*Harada et al., 2017*) (Fig. 3A). This setup allows for the simultaneous application of force and the detection of changes in intracellular cAMP levels. Single cells within a confluent monolayer were chosen for the measurements based on the detection of the GFP signal from the pUltra vector, which allows for bicistronic expression of EGFP and GPR126 (*Lou et al., 2012*) and the fluorescent signal from the Pink Flamindo cAMP sensor (*Harada et al., 2017*) (Suppl. Fig. S3). Since COS-7 cells are not suited for AFM experiments due to their weak adherence to glass coverslips, we used GripTite™ 293 MSR (HEK-GT) cells instead. Receptor cell surface expression and activation in HEK-GT cells was confirmed prior to AFM experiments by ELISA and cAMP accumulation assays, respectively (Suppl. Fig. S4). Relative possible Pink Flamindo fluorescence changes upon stimulation of GPR126 with either forskolin, anti-HA antibody or *Stachel* peptide pGPR126 are displayed in Suppl. Fig. S5.

The course of the cantilever deflection and therefore the force applied to the cell for the pushing setup is depicted in Figures 3B and 3C. The cAMP-evoked changes in the Pink Flamindo

fluorescence were monitored by imaging right before the AFM cantilever applied pressure or a pulling force and immediately after its retraction (Fig. 3B). A cantilever coated with human Fc-protein instead of anti-HA antibodies was used as negative control.

When a pushing force of 1nN was applied over varying times, a significant increase in the Pink Flamindo fluorescence signal could be observed compared to the negative control. This effect got stronger and more significant over time (Fig. 3D: 180s and 300s) suggesting a time-dependent increase in receptor activation (Fig. 3D). The control condition responded with a reduction of cAMP sensor intensity over time, presumably due to bleaching effects by the AFM detection laser. Having established that a significant increase in intracellular cAMP can be achieved by pressure application via anti-HA antibody coated tips, we applied varying forces over a constant time in order to quantify the strength of the pushing force needed to activate GPR126 (Fig. 3E). Applying varying pushing forces (0.25nN - 1.5nN) with anti-HA antibody coated tips over 60s lead to a highly significant increase in cAMP levels consistently over the whole range of applied forces. When we performed the same experiment with the cleavage-deficient mutant H839R (Fig. 3F), which showed no mechano-activation in cAMP accumulation assays using antibodies and dynabeads (Figs. 2C, D), we observed no change in the cAMP-mediated Pink Flamindo signal regardless of the pressure applied. This finding suggested that pushing-mediated mechano-activation also requires autoproteolytic cleavage of GPR126.

To further investigate the type and force of antibody-mediated mechano-activation of GPR126 we used the force-clamp setup, in which a pulling force is applied to the cell and receptors (Figs. 3G, H). An Fc-control antibody-coated cantilever could not be used as a negative control in this case, as it does not bind to the receptor or the HA-epitope and therefore no pulling forces would be applied. Instead we compared the WT receptor to the mechano-insensitive H839R mutant, which was still able to bind the anti-HA antibody. We applied varying pulling forces (0.25 nN - 0.75 nN) over 60 seconds (Fig. 3I) and 300 seconds, as fold changes were very low after the shorter pulling time period (Fig. 3J). We found the pulling forces to significantly activate the receptor and observed a tendency towards lower pulling forces (0.25nN) being more efficient than higher pulling forces (0.75nN).

Figure 3. Mechano-activation of GPR126 via anti-HA antibodies. (A) Schematic AFM experiment setup with a coated tipless cantilever pressing on a cell co-transfected with GPR126 and the Pink Flamindo cAMP sensor. (B, C) Course of the AFM cantilever deflection during the pushing experiments and the forces applied to the

receptor and corresponding laser deflection: (1) Cantilever approaches cell; (2) Point of contact between cantilever and cell; (3) Constant pressure being applied to the cell; (4) Cantilever is retracted from the cell; (5) Bindings between cantilever and cell rupture; (6) All bindings are ruptured and the cantilever is back in its starting position. (D) Changes in Pink Flamindo fluorescence intensity after applying a pushing force of 1 nN on the WT receptor at different time points (effect of time $p = 0.775$, effect of coating $p = 0.0002$, interaction time $\times$ coating $p = 0.065$; two-way ANOVA). (E, F) Changes in Pink Flamindo fluorescence intensity after applying indicated pushing forces for 60s on the WT receptor (E) (effect of force applied $p = 0.878$, effect of coating $p < 0.001$, interaction force $\times$ coating $p = 0.882$; two-way ANOVA) and the H839R mutant (F) (effect of force applied $p = 0.981$, effect of coating $p = 0.0867$, interaction force $\times$ coating $p = 0.924$; two-way ANOVA). (G, H) Course of the AFM cantilever deflection during the force clamp experiments and the forces applied to the receptor and corresponding laser deflection: (1) Cantilever approaches cell; (2) Point of contact between cantilever and cell; (3) Initial pressure being applied to allow binding between cantilever and cell; (4) Cantilever is retracted from the cell until the desired pulling force is applied; (5) Constant pulling force is applied; (6) Cantilever is retracted from the cell completely; (7) Bindings between cantilever and cell rupture; (8) All bindings are ruptured and the cantilever is back in its starting position. Changes in Pink Flamindo fluorescence intensity after applying indicated pulling forces over (I) 60s (effect of force applied $p = 0.297$, effect of receptor variant $p = 0.0135$, interaction force $\times$ receptor variant $p = 0.497$; two-way ANOVA) and (J) 300s (effect of force applied $p = 0.405$, effect of receptor variant $p = 0.0386$, interaction force $\times$ receptor variant $p = 0.804$; two-way ANOVA). Data are given as means $\pm$ SEM of three-five different experiments each measuring 3 individual cells for each force and time. Statistics were performed as 2-way ANOVA followed by Tukey post hoc analysis; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Significances given at the right of each graph show the result of the ANOVA and significances given above individual points in the graphs show the result of the post hoc analysis. Schematic images (A-C and G-H) were created with BioRender.com. The Pink Flamindo depiction in A was taken from (Harada *et al.*, 2017).

Endogenous ligands of GPR126 convey a highly specific type of mechano-activation

Having established a reliable way to measure activation-inducing forces on GPR126, we set to investigate the forces needed to stimulate GPR126 using its natural ligands. Several ligands have been shown to modulate GPR126 activity (Fig. 4A): for example collagen IV has been described as directly activating ligand (Paavola *et al.*, 2014), which could be interpreted as a pushing force on the receptor as it ‘sits’ on it. Laminin 211 on the other hand was reported to require mechanical stimuli such as shaking or vibration to induce cAMP signaling (Petersen *et al.*, 2015), which could be a proxy for pulling forces. To test these assumptions, tipless AFM cantilevers were coated with collagen IV or laminin 211. Again, all experiments were performed on HEK-GT cells co-transfected with Pink Flamindo and either WT GPR126 or the GPR126 H839R mutant. The empty pUltra vector was used as a negative control.

Interestingly, when pushing at WT GPR126 transfected cells with a collagen IV-coated cantilever (Fig. 4B), it took specifically 1 nN to induce a significant increase in cAMP levels (Fig. 4C). Lower or higher pressure did not activate the receptor. Again, GPR126 H839R could not be activated through this mechanical stimulus. Applying pulling forces with collagen IV did not produce any significant changes in the Pink Flamindo fluorescence signal for neither the WT receptor nor the cleavage-deficient mutant (Fig. 4D). The Laminin 211-coated cantilever (Fig. 4E) did not activate GPR126 through pushing (Fig. 4F), but a significant increase in the Pink Flamindo fluorescence signal was seen in the force-clamp setup when applying a pulling force of 0.75 nN (Fig. 4G). Lower pulling forces were not sufficient to activate the receptor.

Figure 4. Mechano-activation of GPR126 via its ligands. (A) The domain architecture of human GPR126 with the binding sites of its ligands, collagen IV and laminin 211, is depicted. (B, E) Schematic demonstrating the AFM setup with collagen IV (B) and laminin 211 (E). (C, D) Changes in Pink Flamindo fluorescence intensity after applying a varying pushing (C) or pulling (D) force over 60s with a collagen IV-coated cantilever. (F, G) Changes in Pink Flamindo fluorescence intensity after applying a pushing (F) or pulling (G) force over 60s with a laminin 211-coated cantilever. Data are given as means $\pm$ SEM of three different experiments, each measuring 3 individual cells for each force. Statistics were performed as 2-way ANOVA (C: Interaction: $p=0.029$; G: Interaction: $p=0.025$; D, F: n.s.) in combination with Tukey post hoc analysis; * $p < 0.05$; ** $p < 0.01$. Significances in the graphs show the results of the post hoc analysis. Schematic images were created with BioRender.

Discussion

The aGPCR GPR126 can be activated through different mechanisms, including agonistic *Stachel* sequence-derived peptides (Liebscher et al., 2014), its ligands collagen IV (Paavola et al., 2014), laminin 211 (Petersen et al., 2015) and prion protein (Küffer et al., 2016), the small

molecule compound apomorphine (*Bradley et al., 2019*) and mechanical stimuli such as vibration or shaking (*Petersen et al., 2015*). Yet, all of these activators lack specificity for GPR126, which hampers their usefulness as tools for *in vivo* experiments or potential therapeutics. The agonistic peptide pGPR126 can for example cross-activate the aGPCR GPR64/ADGRG2 (*Demberg et al., 2017*), while the ECM molecules collagen and laminin can also bind and activate integrins (*Keely et al., 1995*) and apomorphin is an agonist on dopamine receptors (*Millan et al., 2002*). In this study we show that a commonly used monoclonal antibody targeting an N-terminal HA epitope can serve as an activator of GPR126. This activation is further increased by adding a secondary antibody. Paramagnetic Dynabeads® coated with the anti-HA antibody could also stimulate cAMP production in GPR126 transfected cells, however to a similar extend as the anti-HA ab alone, while a magnetic field placed below the cell culture well did not alter this effect. Future studies with a stronger or alternating magnetic field might be able to trigger GPR126 activation.

Antibody-mediated GPCR activation is a known phenomenon. For example, in several disease contexts, autoantibodies target GPCRs, such as the thyroid stimulating hormone receptor, calcium-sensing receptor and muscarinic M1 and M2 receptors (*Unal et al., 2012*). However, here we use antibodies to experimentally modulate aGPCR signaling and create a new toolbox that allows us to study receptor activation. Previously, the aGPCRs EMR2 and GPR56 were shown to be activated through antibodies targeting the receptors' N-termini (*Bhudia et al., 2020; Chatterjee et al., 2021; Yona et al., 2008*). In the absence of known antibodies against the N-terminus of GPR126, we introduced the HA epitope into the N-terminus of GPR126 and employed a commercial anti-HA antibody to modulate signalling. While the effect is elicited using an exogenous epitope and its cognate antibody, it nonetheless illustrates the principle that antibody binding to the N-terminus of GPR126 influences receptor activation, similar to other aGPCRs. Ultimately, development of antibodies against the native amino acid sequences of GPR126 will pave the way towards further basic experimentation and therapeutic applications.

When studying aGPCR activation by ligands or antibodies, splice variants have to be considered, since the complex exon-intron composition results in a large subset of functionally divergent receptors. As an example, activation of GPR56 through an antibody targeting the GAIN domain is dependent on a Serine–Threonine–Proline-rich (STP) region, which otherwise does not influence basal signaling levels of the receptor (*Chatterjee et al., 2021*). In the case of GPR126, alternative splicing influences the domain composition of the N-terminus (*Knierim et al., 2019*). Within the N-terminus, the CUB/PTX domain is of functional relevance as it

serves as point of interaction for collagen IV (*Paavola et al., 2014*), yet, for anti-HA antibody-mediated mechano-activation it appears to be neglectable. This would decrease considerably a potential variability of the antibody-induced response when used in a physiologic setting. In contrast, elongating the receptor's N-terminus through the addition of a fluorescent protein abolishes this activation. Similar observations have been made for the adhesion GPCR dCIRL, where elongation of the N-terminus reduces the response to mechanical stimuli (*Scholz et al., 2017*). Thus, large artificial N-terminal domains influence signaling properties, even when they do not act as binding sites for the activating antibodies. It is currently unknown whether this is due to the simple change in length or altered three-dimensional structure of the N-terminus.

We have further investigated the role of cleavage in antibody-mediated activation of GPR126. The receptor's N-terminus harbors a furin site located in the SEA domain, and an autoproteolytic cleavage motif (GPS) within the GAIN domain. We find that abolishing furin cleavage does not affect response to antibody-mediated activation while abrogation of the GPS cleavage blunts the effect of the antibody. This observation may support the idea that NTF-CTF dissociation is an integral part of the receptor's response to the antibody treatment, and its activation in general. However, this concept may be receptor-specific rather than generalizable to all aGPCRs. For example, antibody-mediated activation of GPR56 (*Chatterjee et al., 2021*) and mechano-sensation of latrophilin (*Scholz et al., 2015*) are not affected by mutations of their GPS cleavage motifs. Thus, it seems that autoproteolysis at the GPS serves distinct purposes among different receptors. Our experiments also demonstrate that antibody-mediated activation of GPR126 depends on the endogenous encrypted agonist, the *Stachel* sequence. This, combined with the finding that the effect requires autoproteolytic cleavage, suggest the underlying mechanism involves high-affinity antibody binding to the N-terminus, induced dissociation of the NTF and CTF, and a CTF configuration that allows the *Stachel* sequence to act as the endogenous agonist.

In many ways, the activation of GPR126 through the anti-HA antibody resembles characteristic features seen for the activation through mechanical forces, such as vibration and shaking, including the dependence on the tethered agonist and cleavage (*Petersen et al., 2015*), and the negative impact of N-terminal elongation (*Scholz et al., 2015*). We, therefore, hypothesize that antibody-induced GPR126 activation mechanistically overlaps with mechano-activation.

While mechano-activation has been widely accepted as a component of aGPCR activation, there are still unanswered questions with regard to the magnitude and type of forces needed. To begin answering these questions in the case of GPR126, we employed an atomic force

microscopy approach in combination with fluorescent cAMP imaging. Using an antibody-coated cantilever, we found that tonic forces, whether pushing or pulling, as little as 0.25 nN induce receptor activation. This might explain, why we did not detect any additional activation when placing a magnet below the cell culture plate, as the Dynabeads® alone could push enough to reach saturating effects. The combination with a secondary antibody could enhance GPR126 activation through an increased cross-linking of receptors, which would combine pushing and pulling forces.

The push and pulling effects depend on autoproteolytic cleavage, similar to the effects of the antibody alone and antibody-coated beads. To ensure that applying pushing forces onto cells is not a non-specific event, we showed that there is no change in cAMP levels of GPR126-expressing cells when the cantilever is coated with an Fc fragment. Even though published single molecule AFM analysis indicated that forces in the pN range are sufficient to unfold GPCRs such as the protease-activated receptor 1 (PAR1) (*Alsteens et al., 2015*), there is no indication that the pulling forces exerted in our experimental paradigm would lead to such a structural disruption, since we use a tipless cantilever that binds multiple antibodies, thereby interacting with several receptors at the same time. As the size of the adhesive interface and traction forces display a linear relationship, one would expect a much higher traction force needed to unfold GPR126 in our setup. A comparable force requirement could be deduced from an experiment that studied the interaction strength between fibronectin-coated beads and cell surface. The forces needed to disrupt this interaction were > 2 nN (*Mierke et al., 2008*), which is much higher than the pulling force (0.25 nN) needed to activate GPR126. Therefore, we conclude that the increase in cAMP is specifically mediated by a pulling force on the intact receptor and does not present an artefact due to structural destruction of the receptor.

These pulling forces are also below the traction forces that are normally transmitted by cell adhesions to the surrounding extracellular matrix (ECM) (1 – 10 nN) (*Balaban et al., 2001*), indicating that even small changes can be detected by an aGPCR as it is embedded within the ECM. The forces mediated by two known ligands of GPR126 that change intracellular cAMP are indeed within this physiologic force range. We found that collagen IV requires a pushing force of 1 nN to raise cAMP, while Laminin 211 activates GPR126 upon pulling with a force of 0.75 nN. Our results also match observations from previous signalling assays, where incubation of GPR126-expressing cells with laminin 211 requires the addition of vibration or shaking to induce an increase in cAMP (*Petersen et al., 2015*). Both activation patterns fit the physiological setting for these ligands in the process of myelination (*Bunge et al., 1990*). Schwann progenitor cells require low cAMP levels to continue proliferation. This proliferation

occurs in the same time window as the production of the first basal membrane components, among them laminin 211. Only when there are sufficient amounts of laminin 211 does it start polymerizing. The polymer, in turn, is believed to exert a traction force on GPR126 leading to a burst of cAMP, which eventually switches the Schwann progenitor cells from the proliferative to a differentiated state and leads to myelination (*Bacallao and Monje, 2015; Petersen et al., 2015*). Collagen IV is then added onto the laminin 211-based membrane to stabilize it. The presence of collagen IV, through its activation of GPR126, maintains the higher cAMP levels associated with Schwann cell differentiation and myelination.

In summary, we were able to define the mechanical forces required to induce ligand and antibody-mediated activation of GPR126 *in vitro*, where the antibody reproduces both ways of mechanical stimulation employed by the endogenous ligands. Our findings emphasize the role of mechano-activation in translating interaction of GPR126 with ECM components to receptor activation and signalling and provide a tool to study these in a simplified but modifiable antibody-based setup.

Materials and Methods

If not stated otherwise, all standard substances were purchased from Sigma Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), and C. Roth GmbH (Karlsruhe, Germany). Cell culture material was obtained from ThermoFisher Scientific (Schwerte, Germany) and primers were obtained from Microsynth Seqlab GmbH (Göttingen, Germany).

The constructs of human GPR126 and Δ CUB, Δ PTX, Δ CUB Δ PTX mutants have been described previously (*Liebscher et al., 2014; Mogha et al., 2013; Petersen et al., 2015*). Point mutations for R468A and H839R mutants were inserted by quick change mutagenesis. In brief, plasmid DNA was amplified with PCR and then digested with DpnI restriction enzyme for 4 h at 37 °C prior heat shock transformation in *E. coli*. The mRuby epitope was inserted in the human GPR126 construct after the hemagglutinin (HA) epitope by a PCR-based site-directed mutagenesis and fragment replacement strategy. The sequences of all generated mutants of human GPR126 were verified by Sanger sequencing (Microsynth Seqlab, Göttingen, Germany).

The GPR126/pULTRA construct was generated using One-step isothermal DNA assembly (*Gibson et al., 2009*). The reaction buffer was prepared according to protocol. All enzymes were acquired from NEB: T5 exonuclease (M0363), Taq DNA Ligase (M0208) and Phusion DNA Polymerase (M0530). The wt receptor DNA for the assembly was obtained via PCR using the previously described construct of the full-length human GPR126 in the pcDps vector (*Liebscher et al., 2014*). The pULTRA (pUltra was a gift from Malcolm Moore, Addgene plasmid #24129, (*Lou et al., 2012*)) vector was restriction digested using XbaI (NEB, R0145).

In vitro functional assays

The human N- and C-terminally tagged GPR126 was heterologously expressed in COS-7 cells grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin or the GripTite 293 MSR Cell Line (ThermoFisher Scientific) (HEK-GT) grown in DMEM supplemented with 10 % fetal bovine serum, 1 % G418 (ThermoFisher, 10131035) and 1 % Non-Essential Amino Acids (ThermoFisher, 11140050) at 37 °C and 5 % CO₂ in a humidified atmosphere. For the establishment of antibody-based assays, conjugated Dynabead stimulation in a magnetic field and enzyme-linked immunosorbent assay (ELISA), cells were split into 48-well plates (3×10⁴ cells/well for COS-7 or 1.3×10⁵ cells/well for HEK-GT) or into 6-well plates for total ELISA

(3×10^5 cells/well for COS-7 or 1.5×10^6 cells/well for HEK-GT). For peptide stimulation, cells were split into 96-well plates (1.5×10^4 cells/well for COS-7 or 4.5×10^4 /well for HEK-GT). For Western Blot analysis, cells were split into 24-well plates (6×10^4 cells/well for COS-7). Transfection was done with Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's protocol. 50 ng (96-well plates) or 500 ng (48-well plates) of receptor plasmid DNA/well were used for cAMP accumulation assays. To perform cAMP accumulation assays, 48 h after transfection, GPR126 and empty vector-transfected cells were stimulated with the indicated concentrations of anti-HA antibody (H3663, stock concentration 1 mg/ml, Sigma-Aldrich) or anti-FLAG antibody (F1804, Sigma-Aldrich) in DMEM for 1 h, followed by incubation with 3-isobutylmethyl-xanthine (1 mM)-containing medium or including the secondary antibody anti-mouse IgG (Fc specific, M2650, Sigma-Aldrich) for 30 min. To perform mechanical forces on transfected COS-7 cells, cells were treated with vibration frequency of 17 Hz for 30 min. For peptide stimulation, *Stachel*-sequence derived peptide was diluted in IBMX-containing medium. Peptide solution from purified powder was achieved by preparing a 100 mM in 100 % DMSO solution, which was further diluted into 10 mM stocks using a 50 mM, pH 8 Tris buffer and finally pH controlled. Peptide concentrations used in assays are 1 mM. The 1 mM peptide solution contains 1 % DMSO and 10 % of Tris buffer used for dilution. After stimulation cells were lysed in LI buffer (PerkinElmer Rodgau, Germany) and kept frozen at -20°C until measurement. To measure cAMP concentration, the Alpha Screen cAMP assay kit (PerkinElmer) was used according to the manufacturer's protocol. The accumulated cAMP was measured in 384-well white OptiPlate microplates (PerkinElmer) with the EnVision Multilabel Reader (PerkinElmer). Super paramagnetic Dynabeads® (14311D, ThermoFisher Scientific) were conjugated with anti HA antibody according to the manufacturer's protocol. The magnetic stimulator was created via embedding N45 NdFeB magnets (https://www.supermagnete.de/data_sheet_S-08-05-N.pdf) into a custom 3D printed holder matching the outer diameter of a corresponding cell culture plate.

To estimate cell surface expression of receptors carrying an N-terminal HA tag, an indirect cellular enzyme-linked immunosorbent assay (ELISA) was used (*Schöneberg et al., 1998*).

The HEK-GT cells used for cAMP accumulation assays and ELISA were not only transfected with the receptor plasmid DNA but the Pink Flamindo cAMP sensor as well to account for changes in expression and activatability due to the co-transfection used in the AFM experiments.

Western Blot

COS-7 cells were transfected with either empty pcDps vector, or GPR126, GPR126 R468A and GPR126 R468A/H839R using the standard protocols described earlier. Every other transfected construct was incubated with primary anti-HA antibody for an hour as explained before. Cells were lysed with the addition of 300 µl of 2x SDS loading dye. After freeze thaw cycling the lysates were run on a 10% SDS-PAGE gel, followed by western blotting. The membranes were blocked for 1h with 5% non-fat dry milk in TBST buffer, washed 3 times with TBST buffer and incubated over night with either primary antibody (rabbit HA and GAPDH antibody, cell signaling). The following day, membranes were again washed with TBST buffer and incubated with the secondary anti-rabbit antibody, which has an HRP conjugation (cell signaling) for 1h at room temperature. This was followed by three additional washing steps with TBST buffer. Membranes were wetted with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) to visualize protein bands, which were imaged using the Bio-Rad Gel Doc Imager.

AFM

For all AFM experiments, HEK-GT cells were cultured at the same conditions as for the *in vitro* functional assays. Cells were seeded on 24 mm glass coverslips (coated with Poly-L-Lysin (Sigma-Aldrich, P4707), (1 % PLL solution incubated at 37 °C for 5 minutes then washed with PBS and dried under a sterile hood) in 6-well plates (1.5×10^6 cells/well) and co-transfected with the cAMP sensor Pink Flamindo (Addgene plasmid #102356) and either empty vector or the given GPR126 construct in the pULTRA vector on the next day using Lipofectamine 2000 according to the manufacturer's protocol. The media was changed to the described culture media ~24h after transfection and AFM measurements took place ~48h after transfection: the coverslips were transferred into a 35mm cell culture dish and washed with DMEM without phenol red 3 three times and placed into AFM coverslip holders. 750µl culture media without phenol red was added and the cells were stored at 37 °C and 5 % CO₂ until right before the measurements took place.

Tipless silicon nitride AFM cantilevers (NanoWorld, PNP-TR-TL) were coated with monoclonal anti-HA antibodies produced in mouse (H3663, Sigma-Aldrich) or Fc-control for the antibody-based mechano-activation experiments using flexible PEG spacers as described before (*Ebner et al., 2007*). Recombinant human Fc was expressed by transfected Chinese hamster ovary (CHO) cells, supernatant was collected and purification was done via His-tag using HisLink Protein Purification Resin (V8823, Promega) according to manufacturer's instructions. Protein purity was confirmed by Western blot analysis. For the ligand-based

mechano-activation experiments, tipless silicon nitride AFM cantilevers were washed twice in Chloroform and dried. The cantilevers were then placed into a collagen IV (C6745, Sigma Aldrich) or laminin-211 (LN221, BioLamina, Sundbyberg, Sweden) solution (0.15 mg/ml) and incubated at 4 °C overnight. The next day the cantilevers were washed in HBSS twice and stored in HBSS at 4 °C until use.

Atomic Force Microscopy (AFM) measurements were performed using a Nanowizard IV AFM (Bruker, Billerica, MA) mounted on an IX 83 inverted optical microscope equipped with a 63x PL APO NA 1.42 oil objective (both Olympus Life Sciences, Wallisellen, Switzerland) and coupled to a X-Cite Exacte Light source (Excelitas Technologies, Waltham, MA).

Cantilevers were calibrated using the thermal noise method according to (*Slattery et al., 2013*). Successfully double-transfected cells were identified by the GFP from the pULTRA vector and the Pink Flamindo fluorescence signal. The cell was then imaged three times in the following order: Brightfield, GFP and Pink Flamindo using a Zyla sCMOS camera (Andor Technology, Belfast, Northern Ireland). The AFM cantilever was then placed centrally on the cell and approached until contacting the cell surface. Proper positioning was verified by a brightfield image and the baseline Pink Flamindo signal was recorded. During stimulation all light sources except the AFM laser were turned off. Immediately after the stimulation was finished, another image of the Pink Flamindo signal was obtained using the same exposure time as before.

For force clamp measurements, the cantilevers were initially pressed onto the cell with a force of 1 nN for 5 seconds in order to allow antibody/ligands and receptor to bind. The cantilever was then retracted (1 µm/s) until the desired clamp force was reached. This value was kept constant for the indicated times before the cantilever was fully retracted.

Extend and Retract length was 15 µm and Extend and Retract speed was 5 µm/s for all experiments.

For analysis, the Pink Flamindo images made before and after the stimulation were compared by measuring the mean intensity of a rectangular area on the stimulated cell using Fiji Image J (*Schindelin et al., 2012*). To account for variations affecting all cells independently from the stimulation, such as bleaching, a rectangular area was measured on five other cells that were not touched by the cantilever but expressed Pink Flamindo. The average of the change in these 5 reference cells was subtracted from the measured change in the stimulated cell to isolate the effect the AFM cantilever stimulation has on the receptor-transfected cell (Suppl. Fig. S3).

To evaluate the mechano-independent changes of the Pink Flamindo fluorescence signal, HEK-GT cells were split into 96-well plates (4.5×10^4 /well) and transfected the following day analog

to the cAMP accumulation assays described earlier. Two days after transfection the media was removed from the wells and 40µl of DMEM without phenol-red were added to each well. Then the GFP signal (transfection control) as well as the Pink Flamindo fluorescence signal were imaged using a Celigo Image Cytometer (Nexcelom Bioscience). Forskolin, pGPR126 and anti-HA ab diluted in DMEM without phenol-red were added to a final volume of 50 µl/well and following concentrations: 10 µM forskolin, 1 µM anti-HA ab and 1 mM pGPR126. The cells were imaged again 60 s after addition of the respective stimulus and the intensity of the Pink Flamindo signal beforehand and afterwards was compared.

Data Analysis

Receptor expression and activation were analyzed using, 1- and 2-way ANOVA as well as t-test as indicated at each figure legend. p values < 0.05 were considered statistically significant (*p<0.05; **p<0.01; ***p<0.001). All statistical analyses were performed by GraphPad Prism version 6.00 for Windows (GraphPad Software, Inc. (La Jolla, USA)) or Microsoft Excel 2016 (Microsoft Corporation (Redmond, USA)).

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Author Contributions

JM, CW, CS, VS, DGP and IL designed the research plan. JF, GS, JM, HK, SB and CW performed the experiments. JF, GS, JM, SB, CW and IL analyzed results. JM, CW and IL wrote the paper with input from all authors. All authors edited and approved of the manuscript.

Competing interests

The authors declare no competing interest.

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