PSPN/GFRα4 has a significantly weaker capacity than GDNF/GFRα1 to recruit RET to rafts, but promotes neuronal survival and neurite outgrowth ☆

Jianmin Yang[a], Maria Lindahl[a], Päivi Lindholm[a], Heidi Virtanen[a], Eleanor Coffey[b], Pia Runeberg-Roos[a], Mart Saarma[a,*]

[a] *Institute of Biotechnology, Viikki Biocenter, University of Helsinki, Viikinkaari 9, P.O. Box 56, FIN-00014 Helsinki, Finland*
[b] *Turku Centre for Biotechnology, Åbo Akademi and Turku University, FIN-20521 Turku, Finland*

Received 5 April 2004; revised 25 May 2004; accepted 2 June 2004

Available online 15 June 2004

Edited by Gerrit van Meer

Abstract Previously, it was shown that the recruitment of RET into lipid rafts by glial cell line-derived neurotrophic factor (GDNF)/GFRα1 is crucial for efficient signal transduction. Here, we show that the mouse GFRα4 is a functional, *N*-glycosylated, glycosylphosphatidylinositol (GPI)-anchored protein, which mediates persephin (PSPN)-induced phosphorylation of RET, but has an almost undetectable capacity to recruit RET into the 0.1% Triton X-100 insoluble membrane fraction. In spite of this, PSPN/mGFRα4 promotes neurite outgrowth in PC6-3 cells and survival of cerebellar granule neurons. As we show that also human PSPN/GFRα4 is unable to recruit RET into lipid rafts, we propose that the mammalian GFRα4 in this respect differs from GFRα1.

Keywords: GFRα4; Persephin; RET; Cell survival; Neurite outgrowth; Raft

1. Introduction

The glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN) are GDNF family ligands (GFLs) involved in the differentiation, development, and survival of neurons. These structurally related neurotrophic factors can support the survival of dopamine neurons and motoneurons, and are thus potential therapeutic agents for neurodegenerative diseases [1]. Each of these neurotrophic factors preferentially binds to one of the glycosylphosphatidylinositol (GPI)-anchored GDNF family receptors α (GFRα). GDNF binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4. The GFL-activated GFRα receptors all signal through the receptor tyrosine kinase RET [1]. In the presence of GDNF, GFRα1 recruits RET into lipid rafts. The recruitment of RET into lipid rafts has previously been shown to be required for downstream signalling, differentiation and neuronal survival [2–4].

The chicken GFRα4 was the first identified receptor for PSPN [5,6]. Later GFRα4 receptors were characterised in human [7], rat [8] and mouse [9]. PSPN does not cross-activate RET through GFRα1 and GDNF does not cross-activate RET through GFRα4 [7,8]. The mammalian GFRα4 receptors all lack the first N-terminal Cys-rich domain, have short C-terminal hinge regions and thereby differ in structure from the other GFRα receptors, including the chicken GFRα4. In spite of this structural difference, the human GPI-anchored GFRα4 promotes PSPN-dependent survival of sympathetic neurons when co-microinjected with *RET* [7].

The structure and expression of the mouse *Gfra4* (*mGfra4*) have been characterised [9], but the functionality of this receptor has not been studied. Here, we characterise the basic biochemical and cell-biological features of this receptor, and show that PSPN/GFRα4 contrary to the previously described GDNF/GFRα1 fails to recruit RET to lipid rafts upon ligand stimulation.

2. Materials and methods

2.1. Constructs, transfections and cell lines

The cDNAs encoding mouse *Gfra4*-FLAG with an N-terminal FLAG-tag and rat *Gfra1* were cloned into pcDNA3. The cDNAs encoding human short isoform of *RET* (in pBabePuro) and human long isoform of *RET* (in pCR3.1) were gifts from Marc Billaud. Neuro 2a cells (ATCC) were transfected with FuGene 6 (Roche Molecular Biochemicals), PC6-3 cells [10] with Lipofectamine 2000 (Invitrogen), and rat primary cerebellar granule neurons with the calcium phosphate method [11]. The stable Neuro 2a cell line expressing rat *Gfra1* was a gift from Marc Billaud and the Neuro 2a cell line expressing human *GFRA4* has been described by Lindahl et al. [7]. The Neuro 2a cell line stably expressing mouse *Gfra4-FLAG* was generated under G418 selection.

2.2. Antibodies

In this work, we used antibodies to FLAG (M2, Sigma, 1:1500), GFRα1 (ΨProSci, 1:500), RET (Santa Cruz Biotechnology Inc., C-19 or C-20, 1:500), phosphotyrosine (4G10, Upstate Biotechnology, 1:1500), and transferrin receptor (Zymed Laboratories Inc., 1:500).

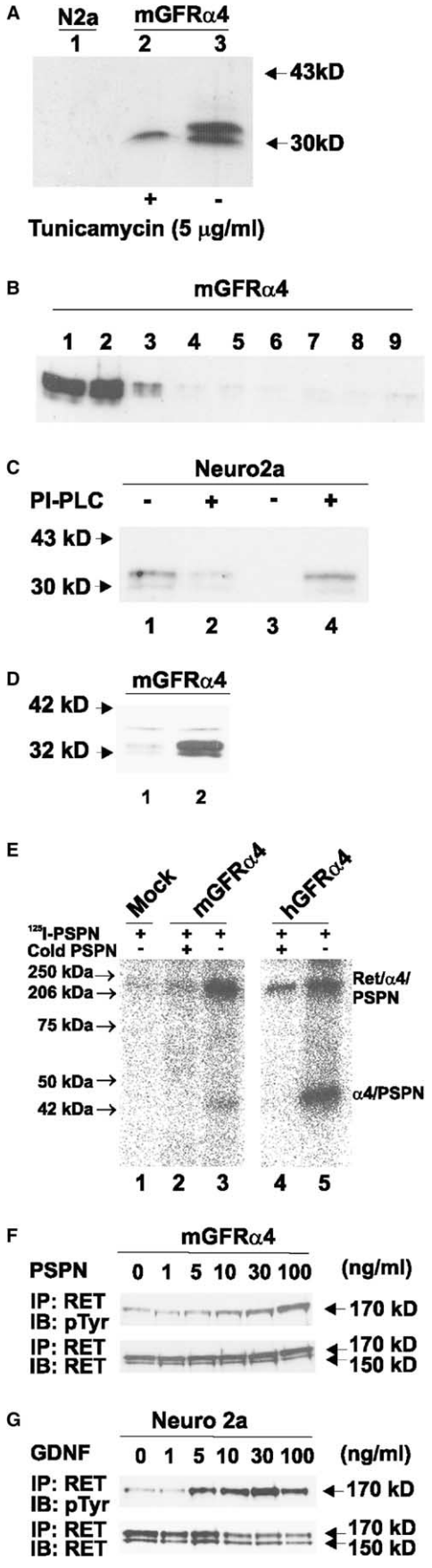
2.3. Glycosylation assay

Stably transfected Neuro 2a cells were cultured with tunicamycin (Sigma) at 5 μg/ml for 16 h. The cells were lysed on ice in lysis buffer (TBS, pH 7.5, 0.2 mM EDTA, 1% Nonidet P-40, 1% Triton X-100,

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2004.06.007.

* Corresponding author. Fax: +358-9-191-59366.
E-mail address: mart.saarma@helsinki.fi (M. Saarma).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.06.007



1 mM PMSF, complete Mini EDTA-free protease inhibitor cocktail, Roche). The post nuclear supernatant (PNS) was analysed by Western blotting with antibodies to FLAG.

2.4. Membrane association assays

Stably transfected Neuro 2a cells were homogenised in 20 mM HEPES, pH 7.5, 0.25 mM sucrose, 1 mM EDTA, 1 mM PMSF, and complete Mini EDTA-free protease inhibitor cocktail, with a syringe. The PNS was mixed with 67% sucrose to give a final concentration of 60% sucrose. The sample was centrifuged in a gradient of 0.5 ml of 67%, 0.5 ml of 60%, 8.0 ml of 50% and 1.0 ml of 5% sucrose (Beckman SW41 rotor; 35 000 rpm; 8 h; +4 °C). Fractions of 0.5 ml were collected from the top and analysed by Western blotting with antibodies to FLAG. The phosphatidylinositol-specific phospholipase C (PI-PLC) treatments were performed with 1 U/ml of PI-PLC (Sigma), for 40 min, in PBS, at 37 °C. Biotinylation of cell surface proteins with Sulfo-NHS-Biotin (Pierce) was done according to the manufacturer's instructions.

2.5. Binding and crosslinking assays

Human PSPN (PeproTech EC Ltd) was ^{125}I-labelled with the lactoperoxidase method and the binding assay as well as crosslinking assay were performed on transiently and stably transfected Neuro 2a cells as described earlier [7].

2.6. Phosphorylation assay of RET

Stably transfected Neuro 2a cells were starved for 4 h in serum-free DMEM at +37 °C and then stimulated with 0–100 ng/ml PSPN for 10 min at +37 °C. Parental Neuro 2a cells were stimulated with 0–100 ng/ml GDNF or PSPN (PeproTech EC Ltd.). RET-associated immunocomplexes were collected with Protein G Sepharose (Amersham Pharmacia Biotech) beads and analysed by Western blotting with antibodies to phosphotyrosine and RET.

2.7. Neuronal survival and neurite outgrowth assays

Cerebellar granule neurons (CGNs) were isolated from postnatal day 7 (P7) rats [12]. On day 5, the cultured neurons were transfected using the calcium phosphate method. After transfection (24 h), the neurons were transferred to serum-free low potassium medium (5 mM) in the presence or absence of PSPN (100 ng/ml). The number of rescued neurons was counted 2.5 days later. As one control, the number of rescued cells was compared to the number of viable cells maintained in high potassium medium (25 mM). As another control, the cells were transfected with *Gfra1* and *RET* and supported by GDNF (100 ng/ml)

Fig. 1. Characterisation of the FLAG-tagged mouse GFRα4 receptor. (A) Western blot analysis of stably transfected Neuro 2a cells. The lysate from naive Neuro 2a cells is shown in lane 1. The FLAG-tagged mGFRα4 is detected with molecular weights of 31 and 33 kD (lane 3). In the presence of tunicamycin, only the 31 kD band of mGFRα4 is detectable (lane 2). (B) Membrane association of the mGFRα4. Membranes from Neuro 2a cells expressing GFRα4 were floated on a sucrose gradient. The fractions were collected and numbered from the top, separated on SDS–PAGE and analysed by Western blot with antibodies to FLAG. (C) Characterisation of the GPI-anchor of the mGFRα4. Western blot analysis of the cell lysates (lanes 1–2) and media (lanes 3–4) from Neuro 2a cells stably expressing the mGFRα4 receptor, before (lanes 1 and 3) or after (lanes 2 and 4) PI-PLC treatment. (D) Biotinylation of cell surface proteins of Neuro 2a cells stably transfected with *Gfra4*. As a control, an extract from cells expressing GFRα4 was precipitated with streptavidin, without any preceeding biotinylation (lane 1). An extract of biotinylated cells expressing the mGFRα4 is shown in lane 2. (E) Crosslinking of ^{125}I-PSPN to Neuro 2a cells transiently transfected with either *mGfra4*-FLAG or human *GFRA4*. After crosslinking, RET was immunoprecipitated from the cell extracts, the precipitate was run on a SDS–PAGE and visualised by autoradiography. (F) Dose-dependent phosphorylation of RET in Neuro 2a cells stably transfected with *mGFRa4*. RET was immunoprecipitated from the cell extracts, and its phosphorylation was monitored on Western blots with antibodies to phosphotyrosine, the blot was reprobed with antibodies to RET. (G) Dose-dependent phosphorylation of RET induced by GDNF in naive Neuro 2a cells. The assay was done as above, except for the induction which was done with GDNF.

in 5 mM potassium. Neurite outgrowth was monitored in transiently transfected PC6-3 cells, 4–5 days after the addition of GDNF or PSPN (100 ng/ml).

2.8. Localisation of RET and mGFRα receptors to lipid rafts

Stably transfected Neuro 2a cells were stimulated with GDNF or PSPN (100 ng/ml) as described for the phosphorylation assay. The cells were collected into cold isotonic lysis buffer and homogenised with a syringe. After addition of 0.1% Triton X-100 to the PNS, the samples were kept on ice for 20 min and analysed on OptiPrep density gradients (0.9 ml 35%, 4.0 ml 30%, and 0.2 ml 0%) as described by Tansey et al. [2]. Fractions of 0.5 ml were collected from the top and analysed by Western blotting.

3. Results

3.1. The mouse GFRα4 is a functional PSPN receptor

Sequence analysis of a putative GFRα4 receptor from mouse predicted a protein of 260 amino acids, harbouring a hydrophobic ER signal sequence, one glycosylation site and a C-terminal GPI-anchor sequence [9]. The predicted molecular weight of this putative mouse PSPN receptor is 29 kD. Our Western blot analysis of Neuro 2a cells, stably transfected with FLAG-tagged *mGfra4*, shows the tagged protein with molecular weights of 31 and 33 kD, respectively. Tunicamycin treatment of the cells shows that the 31 kD band is a non-glycosylated precursor of the glycosylated, mature 33 kD FLAG-tagged mGFRα4 (Fig. 1A).

To study the membrane association of the putative mGFRα4, the cell lysate was analysed on a sucrose gradient, where membranes and proteins bound to the membranes become located in the top fraction. Western blot analysis of the fractions shows that the FLAG-tagged mGFRα4 is located in the top fractions (Fig. 1B). PI-PLC treatment of stably transfected Neuro 2a cells expressing the FLAG-tagged *mGfra4* released the mouse GFRα4 protein from the cell surface and we therefore conclude that the glycosylated 33 kD mGFRα4 is located on the cell surface, where it is bound to the membrane by a functional GPI-anchor (Fig. 1C) The cell surface localisation was further verified by biotinylation of cell surface proteins (Fig. 1D).

To determine whether this putative mouse PSPN receptor directly interacts with PSPN and RET, we used ^{125}I-PSPN and Neuro 2a cells that endogenously express RET and were transfected with mGFRα4. The binding of ^{125}I-PSPN to these cells could be specifically blocked with unlabelled PSPN, and the K_d was 2 nM (data not shown). Crosslinking of cell surface proteins to ^{125}I-PSPN and immunoprecipitation with antibodies to RET revealed two major radioactive bands, which could be displaced with unlabelled PSPN. The molecular weight of these bands could correspond to GFRα4-PSPN and GFRα4-PSPN-RET complexes, respectively (Fig. 1E and Supplementary Fig. 1).

To study if the mGFRα4 receptor mediates signalling via RET, we monitored the phosphorylation of RET in Neuro 2a cells transfected with *mGfra4* and stimulated with PSPN. After immunoprecipitation of the samples with antibodies to RET and staining of the filters with antibodies to phosphotyrosine, we detected a clear dose-dependent phosphorylation of RET (Fig. 1F). PSPN does not mediate phosphorylation of RET in untransfected Neuro 2a cells (Supplementary Fig. 2). GDNF on the other hand mediates a clear and distinct phosphorylation of RET in untransfected Neuro 2a cells, directly purchased from the ATCC (Fig. 1G). The presence of an endogenous GFRα1 receptor and the absence of an endogenous GFRα4 receptor in these cells was verified by PCR (data not shown).

3.2. The GFRα4 receptor recruits RET to rafts very weakly

To set up the flotation assay, we used the previously characterised transferrin receptor, GFRα1, and RET as markers [2]. The transferrin receptor (TFR) was used as a marker for Triton X-100 soluble fractions, whereas GPI-anchored GFRα1 was used as a marker for Triton X-100 insoluble fractions (Fig. 2). Although GDNF/GFRα1 induced a clear re-localisation of RET into the insoluble fraction, PSPN/GFRα4 very weakly recruited RET into this fraction (Fig. 2). As the result was completely unexpected, we included the following controls. First, we verified the PSPN-dependent phosphorylation of RET by splitting the samples and using the other half of them for phosphorylation assays (Fig. 1F, and Supplementary Fig. 3). Second, we confirmed that the addition of the ligands

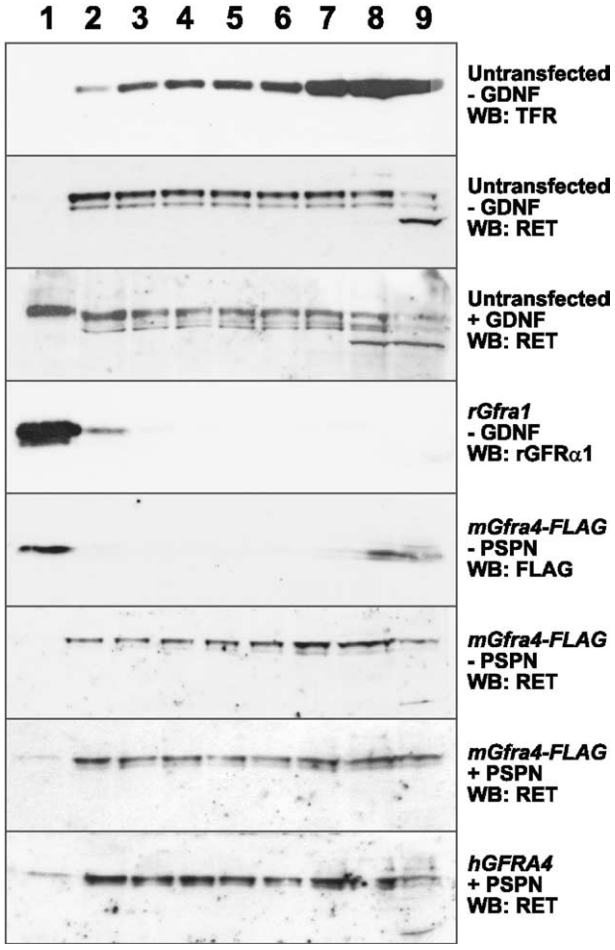


Fig. 2. Density gradient analysis of detergent insoluble membrane fractions. Neuro 2a extracts were analysed in the presence of 0.1% Triton X-100. Parental Neuro 2a cells are marked as untransfected. The transfected construct, the addition of ligand and the antibodies used for Western blotting to detect the GFRα1 receptor, RET, FLAG or transferrin receptor (TFR), are indicated on the right. Nine fractions from each gradient are shown. The Triton X-100 insoluble top fraction is shown on the left as the fraction number 1.

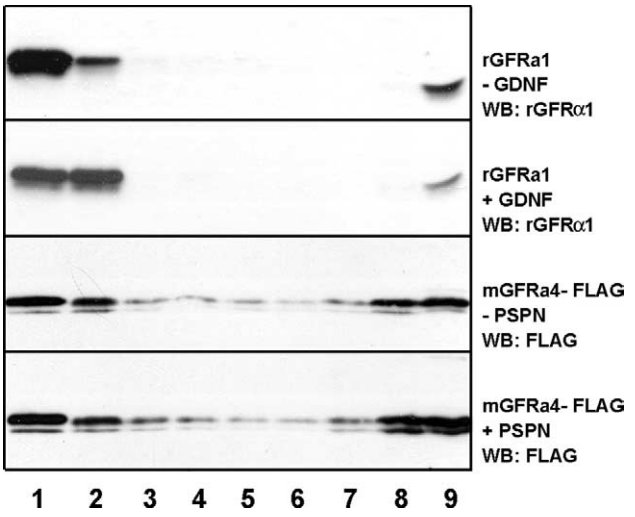


Fig. 3. Density gradient analysis of detergent insoluble membrane fractions. Neuro 2a cell extracts were analysed in the presence of 0.1 % Triton X-100. The transfected construct, the addition of ligand and the antibodies used for Western blotting are indicated on the right. Nine fractions from each gradient are shown. The Triton X-100 insoluble top fraction, is shown on the left as the fraction number 1. The figure shows that the localisation of GFRα1 is unaffected by GDNF and that the localisation of GFRα4 is unaffected by the presence of PSPN.

GDNF/PSPN did not alter the localisation of the GFRα1 or GFRα4 receptors in the gradient (Fig. 3). Third, as the amount of the GFRα1 and the GFRα4 receptors cannot directly be compared with two different antibodies, we instead ensured that the levels of phosphorylated RET were comparable in the samples (Supplementary Fig. 3). Fourth, we confirmed that the N-terminally located FLAG-tag of the mouse GFRα4 receptor did not disturb the protein interactions, by including a Neuro 2a cell line expressing the non-tagged human GFRα4 as a control (Fig. 2). This receptor has previously been shown to be GPI-anchored and to support survival in sympathetic neurons in the presence of PSPN [7]. As the human un-tagged GFRα4 recruited RET to the top fraction as weakly as the mouse FLAG-tagged GFRα4, we conclude that the weak recruitment of RET was not caused by the FLAG-tag.

3.3. *The GFRα4 receptor supports cell survival and mediates neurite outgrowth*

The capacity of mGFRα4 to mediate neuronal survival was tested in rat CGNs. In low potassium (5 mM) and serum-free medium, CGNs undergo apoptosis [13]. We tested if PSPN under these conditions could prevent apoptosis of CGNs transfected with *RET* and *mGFRa4*. Both transfected and untransfected CGNs were viable after 24 h in the medium containing high (25 mM) potassium concentration and serum (K25+S). In the presence of 5 mM potassium and serum-free medium (K5-S), only about 5% of the mock transfected cells survived. PSPN (100 ng/ml) supported neuronal survival only in CGNs that were transfected with both *mGFRa4-FLAG* and *RET*, where it increased the percentage of cell survival to 50% (Fig. 4A).

Previously, it has been reported that PSPN does not have neurite-promoting effects on mature motor neurons [14], but increases neurite outgrowth from oculomotor neurons [15]. Here, we tested the neurite outgrowth inducing capacity of the

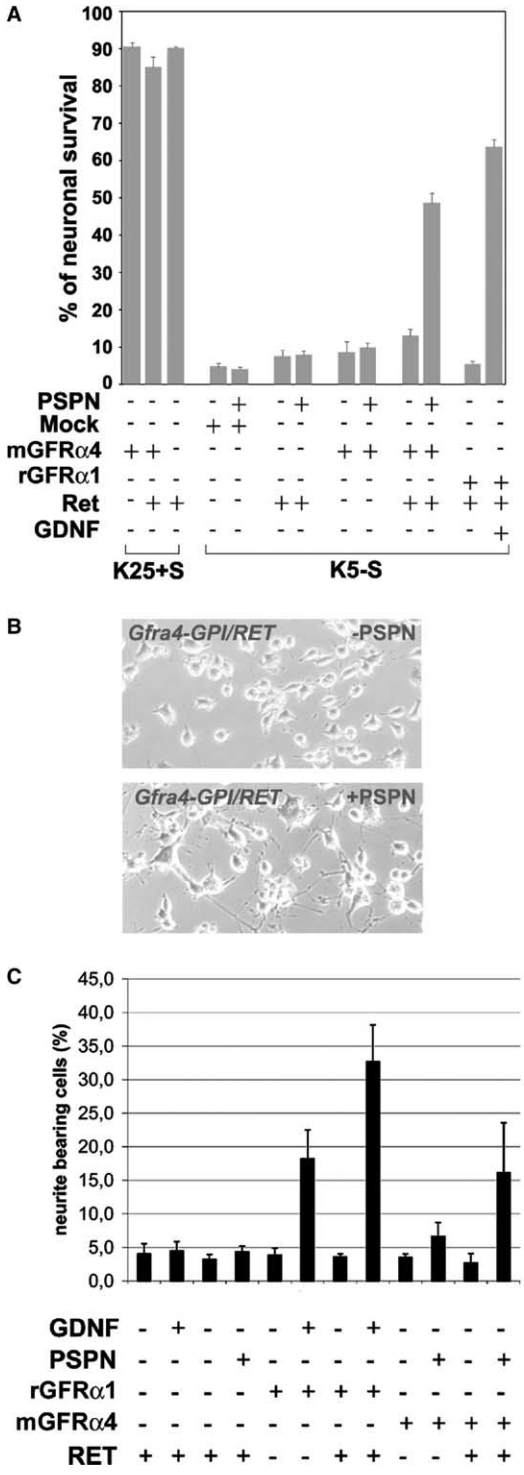


Fig. 4. Neuronal survival and neurite outgrowth assays. (A) Cell survival assay with cerebellar granule neurons transiently transfected with different combinations of *RET*, *mGfra4*, and *rGfra1*. PSPN- or GDNF-dependent cell survival was monitored on transfer to low potassium medium (K5-S). The experiment was repeated three times with parallel samples. An original field of at least 200 transfected cells was used for each sample. The standard errors are indicated with bars. (B) PSPN-dependent neurite outgrowth in PC6-3 cells transiently transfected with *RET* and *mGfra4.* (C) PSPN and GDNF dependent neurite outgrowth assay with PC6-3 cells transiently transfected with different combinations of *RET*, *mGfra4*, and *rGfra1*. The experiment was repeated three times with parallel samples. A field of 200 cells was used for each sample. The standard errors are indicated with bars.

mGFRα4 receptor in transiently transfected PC6-3 cells, which endogenously express RET at a very low level (data not shown). After co-transfection with *Gfra1*/*RET* or *Gfra4*/*RET*, respectively, both GDNF and PSPN induced a clear network of neurites (Fig. 4B and C).

4. Discussion

Here, we show that PSPN/GFRα4 recruits RET to the raft fraction very weakly although PSPN/GFRα4 can stimulate both neurite outgrowth and neuronal survival. Previously, it was suggested that the recruitment of RET into lipid rafts by GDNF/GFRα1 is required for downstream signalling, differentiation and neuronal survival [2].

An artificial transmembrane GFRα1 (GFRα1-TM) was earlier characterised in Neuro 2a cells where it was shown to mediate a strong GDNF-induced phosphorylation of RET, but was located outside the raft fraction. In spite of its full capacity to mediate the phosphorylation of RET, this transmembrane receptor mediated only attenuated neuronal differentiation and survival [2]. However, as we show here that Neuro 2a cells endogenously express GFRα1, which mediates the phosphorylation of RET in untransfected cells, it is evident that the functionality of the GFRα1-TM would need further examinations. The importance of the recruitment of RET into lipid rafts was also previously studied with the aid of a commercially available soluble GFRα1 [4]. The biological activity of this receptor is routinely tested by the manufacturer by assaying its ability to mediate GDNF-induced neurite outgrowth in PC12 cells. Interestingly, the GDNF-activated soluble GFRα1 receptor was inducing a less robust recruitment of RET to lipid rafts than the GDNF-activated GPI-anchored GFRα1 receptor [4].

The GPI-anchored mouse GFRα4 receptor is for some reason not as tightly associated with the raft fraction as the GPI-anchored GFRα1. Because comparing results with different detergents in different cell lines was shown to be difficult [16], we used the same conditions to show that the localisation of the two different GFRα receptors, as well as their capacity to re-localise RET in the Triton X-100 soluble/insoluble fractions differ. The localisation of proteins in different fractions has been proposed to depend not only on protein–lipid interactions, but also on protein–protein interactions [17]. As the structure of the mammalian GFRα4 significantly differs from GFRα1, we propose that GFRα1 and GFRα4 interact differently with cell surface proteins. Thus, it is possible that our results with the two GPI-anchored GFRα receptors reflect their differential interaction with other cell surface proteins.

With the similar method which we used here, phosphorylated RET has been detected both outside and inside the raft fraction after GDNF/GRFα1 stimulation [4]. Therefore, it is evident that we in our work with GFRα4 could not use the localisation of phosphorylated RET as an indication of whether PSPN/GFRα4 activates RET inside or outside the rafts. Moreover, the general significance of the content of different detergent insoluble membrane fractions has recently been very thoroughly questioned [18]. In light of our results, it is at least clear that a robust recruitment of RET to the lipid rafts cannot always and tightly be correlated with the biological activity of a GFRα receptor.

Acknowledgements: We thank Satu Åkerberg and Mari Heikkinen for excellent technical assistance, Johan Peränen for helpful discussions during the work and Timo Päivärinta for rapid processing of the figures. This work was supported by the Academy of Finland, the Sigrid Jusélius Foundation and the EU Grant QLG3-CT-2002-01000.

References

[1] Airaksinen, M.S. and Saarma, M. (2002) Nat. Rev. Neurosci. 3, 383–394.
[2] Tansey, M.G., Baloh, R.H., Milbrandt, J. and Johnson Jr., E.M. (2000) Neuron 25, 611–623.
[3] Encinas, M., Tansey, M.G., Tsui-Pierchala, B.A., Comella, J.X., Milbrandt, J. and Johnson Jr., E.M. (2001) J. Neurosci. 21, 1464–1472.
[4] Paratcha, G., Ledda, F., Baars, L., Coulpier, M., Besset, V., Anders, J., Scott, R. and Ibáñez, C.F. (2001) Neuron 29, 171–184.
[5] Enokido, Y., de Sauvage, F., Hongo, J.A., Ninkina, N., Rosenthal, A., Buchman, V.L. and Davies, A.M. (1998) Curr. Biol. 8, 1019–1022.
[6] Thompson, J., Doxakis, E., Piñon, L.G., Strachan, P., Buj-Bello, A., Wyatt, S., Buchman, V.L. and Davies, A.M. (1998) Mol. Cell. Neurosci. 11, 117–126.
[7] Lindahl, M., Poteryaev, D., Yu, L., Arumäe, U., Timmusk, T., Bongarzone, I., Aiello, A., Pierotti, M.A., Airaksinen, M.S. and Saarma, M. (2001) J. Biol. Chem. 276, 9344–9351.
[8] Masure, S., Cik, M., Hoefnagel, E., Nosrat, C.A., Van der Linden, I., Scott, I.R., Van Gompel, P., Lesage, A.S., Verhasselt, P., Ibáñez, C.F. and Gordon, R.D. (2000) J. Biol. Chem. 275, 39427–39434.
[9] Lindahl, M., Timmusk, T., Rossi, J., Saarma, M. and Airaksinen, M.S. (2000) Mol. Cell. Neurosci. 15, 522–533.
[10] Pittman, R.N., Wang, S., DiBenedetto, A.J. and Mills, J.C. (1993) J. Neurosci. 13, 3669–3680.
[11] Craig, A.M. (1998) Culturing Nerve Cells, 2nd ed. The MIT Press, Cambridge, MA. pp. 79–111.
[12] Miller, T.M. and Johnson Jr., E.M. (1996) J. Neurosci 16, 7487–7495.
[13] D'Mello, S.R., Galli, C., Ciotti, T. and Calissano, P. (1993) Proc. Natl. Acad. Sci. USA 90, 10989–10993.
[14] Bilak, M.M., Shifrin, D.A., Corse, A.M., Bilak, S.R. and Kuncl, R.W. (1999) Mol. Cell. Neurosci. 13, 326–336.
[15] Chen, J., Butowt, R., Rind, H.B. and von Bartheld, C.S. (2003) Mol. Cell. Neurosci. 24, 41–56.
[16] Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A. and Simons, K. (2003) Proc. Natl. Acad. Sci. USA 100, 5795–5800.
[17] McCabe, J.B. and Berthiaume, L.G. (2001) Mol. Biol. Cell. 12, 3601–3617.
[18] Munro, S. (2003) Cell 115, 377–388.