

Study of GDNF-Family Receptor Alpha 2 And Inhibitory activity of GDNF-Family Receptor Alpha 2b (GFR α 2b) isoform

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Abstract- The glial cell-line derived neurotrophic factor (GDNF) and neurturin (NTN) belong to a structurally related family of neurotrophic factors. NTN exerts its effect through a multi-component receptor system consisting of the GDNF family receptor alpha 2 (GFR α 2), proto-oncogene RET and/or NCAM. GFR α 2 is spliced into at least three isoforms, GFR α 2a, GFR α 2b and GFR α 2c. The present study investigated the expression and functional differences of GFR α 2 isoforms. These receptor isoforms are differentially expressed in specific human brain regions. Using Neuro2A model, GDNF and NTN promote neurite outgrowth via GFR α 2a and GFR α 2c, but not GFR α 2b. These GFR α 2 isoforms regulate different early response genes when stimulated with GDNF and NTN. Interestingly, using co-expression models, GFR α 2b inhibits ligand induced neurites outgrowth of GFR α 2a and GFR α 2c, and also the related receptor, GFR α 1a. More intriguingly, ligands activated GFR α 2b was also able to attenuate neurite extension induced by an unrelated stimulation using retinoic acid. MAPK activation induced by GDNF was not attenuated by GFR α 2b in a co-expression model, while the early response genes expression profile (up-regulation of FosB) was similar to that induced by GFR α 2b alone. This study suggest that GFR α 2b is not merely a dominant negative isoform, but signals through a yet to be determined mechanism to antagonize and inhibit neuritogenesis. Together, these data suggest a new paradigm for the regulation of growth factor signaling and neurite outgrowth via an inhibitory splice variant of the receptor.

Key words- GDNF, NTN, GFR α 2, neurites outgrowth. For contact, email: bchtoohp@nus.edu.sg

I. INTRODUCTION

Neurturin (NTN) and glial cell line-derived neurotrophic factor (GDNF) belongs to the GDNF family of neurotrophic factors which are secreted, dimeric proteins with a cysteine knot structure and may be useful as therapeutics for various neurodegenerative diseases including Parkinsons Disease [1]. NTN signals through its preferred receptor GFR α 2 and coupling with Ret and NCAM [2-5]. Although they are structurally related, NTN differs from GDNF in their distribution, and receptor localization [6, 7]. *In vivo*, the chronic administration of NTN produced specific neurochemical changes only in ventrolateral striatum with no detectable

adverse effects, while administration of GDNF resulted in weight loss and allodynia, suggesting functional differences between these two factors [8].

Numerous studies have shown that alternative splicing is prevalent in metazoan genomes, as a means of producing functionally diverse polypeptides from a single gene. A recent genome-wide microarray analysis has estimated that greater than 74% of human multi-exon genes are alternatively spliced [9]. We and others have previously identified 2 other mammalian splice variants of GFR α 2a, GFR α 2b and GFR α 2c [10, 11]. In addition to the existence of spliced isoforms of GFR α 2, multiple isoforms of GFR α 1 [12-14] and GFR α 4 [15-17] have also been reported. The alternatively spliced variants of the co-receptors, RET and NCAM have been shown to have distinct biochemical and physiological functions. These observations are consistent with the emerging view that the combinatorial interactions of the spliced isoforms of GFR α , RET [18-20] and NCAM [21, 22], may contribute to the multi-component signaling system to produce the myriad of observed biological responses.

In this study, the hypothesis that these isoforms have differential signaling mechanisms and functional consequences was tested. In brief, GFR α 2 isoforms mediate neurite outgrowth differently and regulate distinct expression profiles of early response genes. A significant observation is that GFR α 2b, was able to antagonize the ligand induced neurite extension due to GFR α 2a and GFR α 2c, and also inhibits neurite extension induced by retinoic acid.

II. MATERIALS AND METHODS

A. Generation of Neuro2A cells expressing GFR α 2 isoforms

The murine neuroblastoma cell line Neuro2A which express endogenous RET and NCAM, was grown in 10% fetal bovine serum (FBS, Hyclone USA), at 37°C in 5% CO₂. Neuro2A cells were stably transfected with either GFR α 2a, GFR α 2b, GFR α 2c or pIRESneo (Clontech) vector control using Fugene-6 (Roche Lifescience) and selected with 0.8 mg/ml of G418, over a period of 2

months. Primers used for measuring GFR α 2 isoforms expression were as previously described [23]. All subsequent studies were repeated with 3 individual clones.

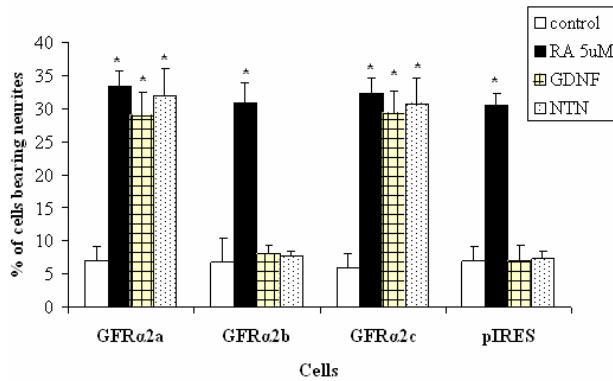


Fig.1 Ligand- induced differentiation of Neuro2a stably expressing GFR α 2 isoforms. Cells treated with retinoic acid (RA), GDNF or NTN (50 ng/ml) for 3 days. Percentage of cells bearing neurites which were twice the length of the cells body was scored. Similar results were repeated with 3 separate clones. Statistical significance was calculated between ligand stimulated and control conditions using paired Students t-test. A value of $P < 0.05$ was considered significant (* $P < 0.002$).

B. Assessment of differentiation in GFR α 2 cells induced by GDNF or NTN

Twenty thousand cells per well were seeded on 6 wells plate over night, at 10% serum DMEM. Cells were then incubated with 0.5% serum media, with or without GDNF or NTN (50 ng/ml). Cells were then incubated for further 3 days. Retinoic Acid (5 μ M) was used as a positive control for inducing differentiation. Percentages of differentiation were counted for cells bearing neurites twice the length of the cells body. More than 600 cells from three random areas were counted per well. Significant differences in percentage of differences between ligand stimulated and control were calculated using paired Students t-test. A value of $P < 0.05$ was considered significant.

C. Analysis of MAP Kinase (Erk1/2) phosphorylation

Phosphorylation of Erk1/Erk2 (Erk1/2) was analyzed as follows. Cells were seeded in DMEM with 10% FBS for 24 h, followed by serum depletion (0.5% FBS) for 16 h. The cells were then treated with 50 ng/ml of GDNF, NTN, Artemin or Persephin (PreproTech, England) in serum free media for different time course at 37 $^{\circ}$ C. For dose response studies, cells were stimulated with different concentration of ligands for 10 min in 37 $^{\circ}$ C. Control treatment with 1 M Sorbitol (Sigma) was carried out simultaneously. The supernatants were removed and cells were washed once with phosphate-buffered saline (PBS) and subsequently lysed in 2% SDS. Protein concentrations were determined using BCA (Pierce, Rockland USA). Western blot using phospho-

specific antibodies according to the manufacturer's instructions (Cell Signaling Technologies). Blots were stripped and reprobbed with actin (Dako) antibodies to verify equal loading of protein.

D. Measurements of early response genes regulated by GDNF and NTN

Cells were seeded in DMEM with 10% FBS for 24 h, followed by serum depletion (0.5% FBS) for 16 h. The cells were then treated with 50 ng/ml of GDNF and NTN (PreproTech, England) in serum free media for different time course at 37 $^{\circ}$ C. To terminate, total RNA isolation and reverse transcription were performed as described above. Quantitative real time PCR were performed as mentioned above, using specifically designed primers, in parallel with plasmid as standard. The fold changes in the target gene, normalized to GAPDH and relative to the expression in control sample.

III. RESULTS

A. GFR α 2 isoforms induced different morphological changes

Neuro2A is a mouse neuroblastoma that does not express GFR α 1 or GFR α 2, but express Ret and NCAM, the co-receptors of GFR α s (data not shown). Hence, we have stably transfected Neuro2A with GFR α 2a, GFR α 2b, or GFR α 2c to generate a cell model to study the functions of GFR α 2 isoforms. GDNF and NTN (50 ng/ml) were able to induce neurites outgrowth in Neuro2A cells via GFR α 2a and GFR α 2c, but not in GFR α 2b (Fig.1). Retinoic acid served as a positive control for neurite outgrowth in all the three GFR α 2 isoforms transfected cells. The percentage of cells bearing neurites in ligand-induced GFR α 2a and GFR α 2c were comparable to those induced by retinoic acid.

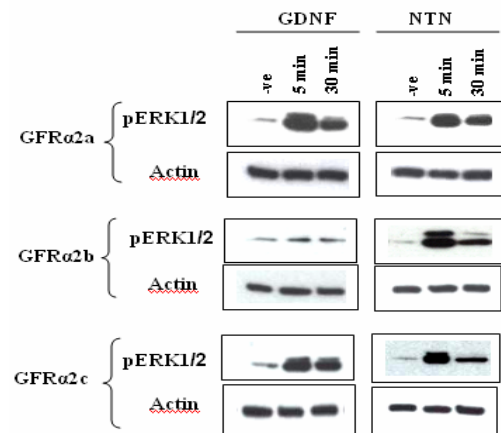


Fig.2 Ligand induced activation of ERK1/2 in Neuro2a cells stably expressing GFR α 2 isoforms. Cells were treated with ligand with designated time points, phosphorylation of ERK1/2 were detected by Western blot analysis. Equal loading were controlled by reprobe for actin.

B. GDNF and NTN showed some selectivity in MAPK activation on GFR α 2 isoforms

NTN phosphorylated ERK1/2 equally well in all three GFR α 2 isoform transfected cells (Fig.2). However, GDNF was only able to induce ERK1/2 activation in GFR α 2a and GFR α 2c, but not GFR α 2b. At supramaximal dose of GDNF (200 ng/ml), ERK1/2 was activated only in GFR α 2a and GFR α 2c transfected cells (data not shown). Interestingly, radioactive labeled GDNF binding studies showed that all GFR α 2 isoforms bound GDNF with similar affinities (data not shown).

TABLE 1
REGULATION OF EARLY RESPONSE GENES

Ligand:	GDNF	NTN
Receptor Isoform:		
GFR α 2a	egr1, Zfp393	egr1
GFR α 2b	<u>fos-B</u>	<u>fos-B</u>
GFR α 2c	egr1, egr2	egr1, egr2

C. GFR α 2 isoforms regulates distinct early response genes

To further understand the mechanisms underlying the activation of GFR α 2 isoforms, we investigated the regulation of some early response genes or transcription factors in ligand activated GFR α 2 isoforms. The changes in expression of genes from fos family (c-fos, fos-B), jun family (c-jun, jun-b), egr family (egr1-4), Zinc finger protein 393 (Zfp393), and GDNF inducible transcription factors mGIF and mGZF1 in GDNF and NTN treated GFR α 2 isoforms were studied. As summarized in Table 1, GFR α 2 isoforms regulated different sets of early response genes. The expressions of these genes were up-regulated and peaked (5 to 10 fold increment) at 1 hour post ligand treatment, and gradually declined for the next 6 hours.

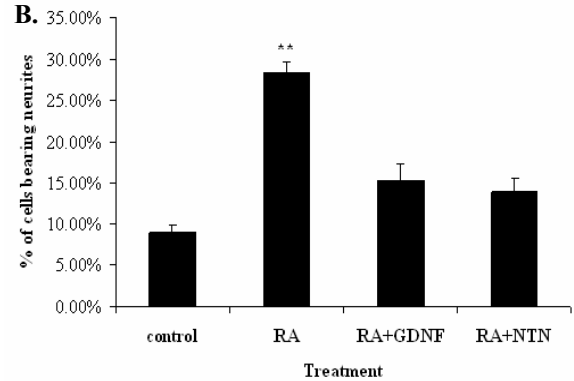
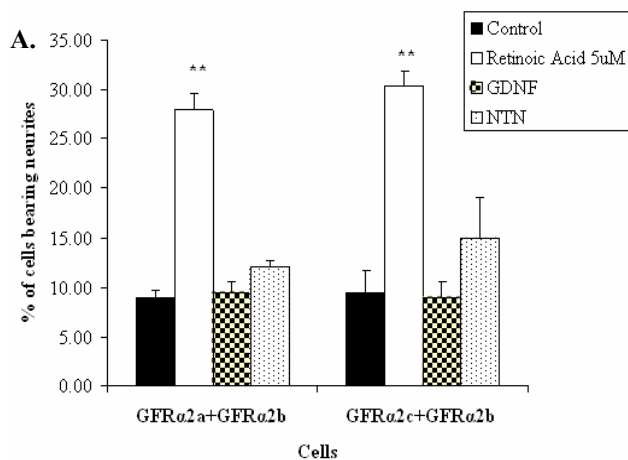


Fig. 3 Studies of neuritogenesis inhibition of GFR α 2b. (A.) Using co-expression model, GFR α 2b antagonizes ligand induced neurites outgrowth of GFR α 2a and GFR α 2c. (B.) Ligand activated GFR α 2b were able to attenuates neurites outgrowth induced by retinoic acid (RA) (5 μ M).

D. GFR α 2b antagonizes neurites outgrowth of retinoic acid and ligand activated GFR α 2a and GFR α 2c

To understand the potential combinatorial functions of GFR α 2 isoforms, we examined the effects of GFR α 2b on neurite extension due to the other GFR α 2 isoforms in a co-expression model. GFR α 2b was cloned into the downstream EMCV IRES driven multiple cloning, while GFR α 2a or GFR α 2c were cloned into the upstream multiple cloning driven by a CMV promoter. Intriguingly, GFR α 2b inhibited the neurite extensions induced by GFR α 2a and GFR α 2c (Fig.3a). Retinoic acid induced differentiation in all the cell lines, verifying that transfection did not affect the abilities of these cells to differentiate. Next, the possibility that GFR α 2b may attenuate neurites outgrowth induced by non-GFR α stimuli was investigated. Using the co-expression cell model, GDNF and NTN activated GFR α 2b and resulted in the inhibition of neurite outgrowth induced by retinoic acids (Fig. 3b).

We further investigated the effects of GFR α 2b on signaling and the regulation of early response gene expressions induced by GFR α 2a/ 2c. Ligand induced MAPK (ERK1/2) activations were not affected in the co-expression model (Fig. 4). However, profiles of ligand induced early response genes regulation were that induced by GFR α 2b activation (fosB was up-regulated) (Table 2).

TABLE 2
REGULATION OF EARLY RESPONSE GENES IN CO-EXPRESSION CELL LINES

Ligand:	GDNF	NTN
Receptor Isoform:		
GFR α 2a + GFR α 2b	<u>fos-B</u>	<u>fos-B</u>
GFR α 2c + GFR α 2b	<u>fos-B</u>	<u>fos-B</u>

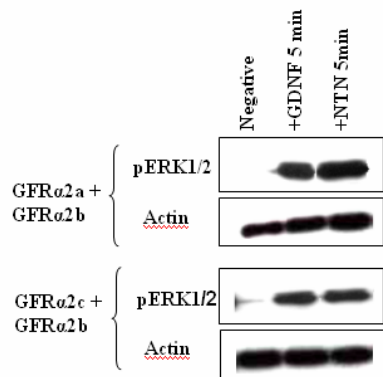


Fig. 4 Study of ERK1/2 activation in GFR α 2 isoforms co-expression model. Neuro2A cells stably co-expressing GFR α 2 isoforms were treated with ligand for 10 min. ERK1/2 phosphorylation was analyzed by Western blot. Equal loading of protein were confirmed with reprobe for Actin.

IV. DISCUSSION

Alternative RNA splicing is a commonly used strategy for creating a functionally diverse pool of gene products derived from a single gene. The emerging view is that the truncated spliced isoforms may act as an inhibitor or regulator for the activity full-length isoforms. During our investigations on the functions of GFR α 2 isoforms, we made several observations that led to unexpected insights into the inhibitory activity of GFR α 2b on its isoforms counterparts.

We have previously shown that GFR α 2 isoforms are differently expressed in murine brain and peripheral tissues[23]. These suggested that all three GFR α 2 isoforms may have significant and distinct physiological role in vivo. In this study, we have shown that GFR α 2 isoforms have different activation mechanism and distinct functional consequences. More importantly, we have shown that GFR α 2b antagonizes GFR α 2a and GFR α 2c.

Dominant negative activity of receptor splice variants have been recently shown in other systems, such as Glucocorticoid receptor [24], Gonadotropin releasing hormone receptor [25] and PPAR receptor [26]. However, unlike these dominant negative isoforms, we have now shown that MAPK activations and fosB up-regulations in GFR α 2b isoform and that this isoform was also able to attenuate differentiation induced by non- GFR α 2 stimuli like retinoic acid.

These indicate that GFR α 2b is not merely a dominant negative isoform, but more likely to activate events that have extensive inhibitory activities. GFR α 2 might be utilizing a yet to be identified mechanism in its antagonism and neuritogenesis inhibition. The mechanism and signaling pathway(s) involved in GFR α 2b inhibitory activity is currently being investigated. In addition, the potential of GFR α 2b in

inhibiting the differentiation induced by other growth factors such as NGF and BDNF will be studied.

V. CONCLUSION

GFR α 2 isoforms have significant functions, and activation mechanisms. We have shown that GFR α 2b is an inhibitory isoform, which antagonizes GFR α 2a and GFR α 2c. Furthermore, ligand activated GFR α 2b was able to attenuate retinoic acid induced neuritogenesis. Together, these data suggest a new paradigm for the regulation of growth factor signaling and neurite outgrowth via an inhibitory splice variant of the receptor.

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