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GDNF-induced cell signaling and neurite outgrowths are differentially mediated by GFRalpha1 isoforms

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) transduces signal and promotes neurite outgrowths in diverse neurons through the interactions of GDNF family receptor alpha 1 (GFRα1) and other co-receptors including Ret receptor tyrosine kinase and NCAM. GFRα1 is alternatively spliced into two isoforms, GFRα1a and GFRα1b, with five amino acids difference. In this study, we found that both GFRα1a and GFRα1b were expressed in various human tissues. Interestingly, when stimulated with GDNF, GFRα1a but not GFRα1b promoted neurite outgrowth in neuroblastoma cells through the activations of ERK1/2, Rac1 and Cdc42. Remarkably, in cells co-expressing GFRα1a and GFRα1b, GDNF inhibited neurite outgrowths. The inhibitory activity of GFRα1b was dependent on RhoA and ROCK activation. Furthermore, GFRα1b but not GFRα1a activated Rho and various ROCK downstream effectors LIMK1/2, cofilin and MLC2. This study demonstrates the hitherto unrecognized roles of GFRα1 isoforms in the activation of distinct signaling pathways and in neurite outgrowths.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a cysteine-knot protein and belongs to the structurally related family of neurotrophic factors (GDNF family ligands, GFLs), which includes Neurturin (NRTN), Artemin and Persephin (Airaksinen and Saarma, 2002). GDNF and its family member NRTN showed potent neuroprotective and restorative effects on dopaminergic nigral neurons, and hence are potential therapeutics for Parkinson's disease (reviewed in Deierborg et al., 2008; Lindvall and Wahlberg, 2008; Marks et al., 2008).

GDNF is known to signal through a multi-component receptor system consisting of the glycosylphosphatidyl–inositol (GPI)-linked receptor GFRα1, and the co-receptor Ret or NCAM (Sariola and Saarma, 2003; Takahashi, 2001; Paratcha et al., 2003). The GDNF receptor complex can recruit various signaling modules and result in distinct cellular outcomes including proliferation, differentiation, survival and motility (Kodama et al., 2005). Since both GFRα1 and Ret genes are alternatively spliced (Dey et al., 1998; Tahira et al., 1990), the diverse and pleiotropic roles of GDNF may be mediated at least partly through the differential expressions and functions of alternatively spliced isoforms of the GDNF receptor complex. GFRα1 is organized into three homologous cysteine-rich domains (Airaksinen et al., 1999). Currently, two alternatively spliced GFRα1 isoforms, GFRα1a and GFRα1b have been identified (Dey et al., 1998; Shefelbine et al., 1998). These two isoforms are highly homologous, with a difference of only five amino acids (140DVFQQ144), which are absent in GFRα1b. These 5 amino acids reside in the N-terminal Domain 1 (D1). Although domains 2 (D2) and 3 (D3) but not D1 are involved in ligand binding, direct chemical cross-linking and proteomic analyses of ligand–receptor interactions showed that the residues at the distal end of the D1 contacted RET at multiple sites and strongly support the biological relevance of the N-terminal D1 (Amoresano et al., 2005). Therefore, it is not unreasonable that 5 amino acids in D1 may modulate the interactions of GFRα1 isoforms with GFLs and co-receptors. To date, most of the studies involving GFRα1 were conducted with GFRα1a and the contribution of GFRα1b to the observed activities of GDNF is unclear.

GDNF, the preferred ligand of GFRα1, has been shown to induce neurite outgrowth and branching morphology in various primary cultures and explants of sympathetic neurons (Trupp et al., 1995), midbrain dopaminergic neurons (Pong et al., 1997), motoneurons (Zurn et al., 1996), sensory neurons (Gavazzi et al., 1999), enteric neurons (Schafer and Mestres, 1999) and similar effects were observed *in vivo* (Kirik et al., 2004; Love et al., 2005). GDNF has also been shown to promote morphological and biochemical differentia-

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF family receptor alpha-1; NRTN, neurturin; RA, retinoic acid; FK, forskolin; LIMK, LIM kinase; MLC, myosin light chain.

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tion in many neuroblastoma cell lines (Hiwasa et al., 1997; Ishida et al., 2007; Park and Jeong, 2006). However, the relative contributions of GFRα1 isoforms to the observed phenotypes in these studies are currently unknown. Interestingly, GFRα1a, either acting in *cis* or in *trans* was able to mediate GDNF-induced neurite formation and axonal growth in both clonal cell lines (Chen et al., 2001; Crowder et al., 2004) and primary neurons (Ledda et al., 2002; Paratcha et al., 2001). Although it has been reported that GFRα1b exhibited distinct biochemical properties from GFRα1a (Charlet-Berguerand et al., 2004; Yoong et al., 2005), the role of GFRα1b in GDNF-induced neurite outgrowth remains to be elucidated.

Here, we report the distinct roles of GFRα1 isoforms in the regulation of GDNF-induced neurite outgrowth. GFRα1a but not GFRα1b induced neurite outgrowth when stimulated by GDNF or NRTN, while GFRα1b inhibited neurite outgrowth of GFRα1a when co-expressed. Furthermore, we showed that these receptor isoforms modulate different RhoGTPase pathways. GFRα1a promotes neurite outgrowth via activation of ERK1/2 and Rac1/Cdc42, while the inhibitory activity of GFRα1b is RhoA-ROCK dependent. These findings not only reveal the distinct cellular functions of alternatively spliced GFRα1 isoforms, it also suggests that inhibitory activity of GFRα1b may serve as a regulatory switch in neuritogenesis and contribute to the functional complexity of GDNF signaling.

Results

GFRα1a and GFRα1b isoforms are expressed in human tissues and the central nervous system

To gain a better understanding of the possible physiological relevance of GFRα1 isoforms, we measured the gene expression profiles of GFRα1 isoforms in various human tissues and different regions of central nervous system (CNS) (Fig. 1). We have previously designed highly specific and sensitive quantitative real time PCR assays for the quantification of mouse GFRα1 isoforms (Yoong et al., 2005). Using a similar approach, we found that GFRα1b was the dominant isoform expressed in all the peripheral tissues examined (Fig. 1A). Interestingly, GFRα1b was found to be expressed at exceptionally high level in the adult liver, similar to that previously reported in mouse adult liver (Yoong et al., 2005). In the substantia nigra and thalamus, GFRα1a was found to be expressed at significantly higher levels than GFRα1b, while the reverse was observed with the expression of GFRα1b in the caudate nucleus, cerebral cortex, putamen, subthalamic nucleus, and spinal cord (Fig. 1B). Due to the lack of GFRα1 isoform-specific antibodies, it is unknown whether both GFRα1a and GFRα1b proteins are expressed in same cells. Together, these data showed the differential expression profiles of GFRα1 isoforms, with significant GFRα1b expressions in all human tissues and various regions of the CNS examined.

Ligand activated GFRα1 isoforms induced ERK1/2 and AKT activations and the expressions of different early response genes

We have previously generated Neuro2A transfectants stably expressing similar levels of GFRα1a or GFRα1b (Yoong et al., 2005) and examined the acute activation of ERK1/2 in these cell lines by GDNF and NRTN. In this study, we extended the ligand treatment for a period of 3 h to examine the kinetic of ERK1/2 as well as AKT activation. GDNF or NRTN treatment in both cell lines induced transient phosphorylation of ERK1/2 (Fig. 2A). Both ligands were found to induce sustained phosphorylation of AKT in Neuro2A cells expressing GFRα1a or GFRα1b (Fig. 2A). In an effort to further understand the functions of these isoforms, we examined the changes in the expressions of some early response genes which have previously been shown to be regulated by GDNF or NRTN (Fukuda et al., 2003; Morinaga et al., 2005; Pezeshki et al., 2003; Yajima et al., 1997). Activation of GFRα1a by GDNF was found to significantly up-regulate the expressions of *egr-1*, *egr-2*, *c-fos* and *fosB* (Fig. 2B). Similarly, NRTN up-regulated *egr-1* robustly with lesser increase in the expressions of *egr-2* and *fosB* in GFRα1a expressing cells (Fig. 2B). However, GDNF and NRTN only up-regulated the expression of *egr-2* in GFRα1b expressing cells (Fig. 2B). No significant changes in the expressions of *c-jun*, *jun-b*, *egr 3*, *egr 4*, *mGIF* and *mGZF1* were observed in response to GDNF or NRTN in either GFRα1a or GFRα1b expressing cells (data not shown). These results showed that the activation of GFRα1 isoforms results in distinct transcriptional activation of specific early response genes.

GFRα1a but not GFRα1b induced neurite outgrowths. The neurite outgrowth process is dependent on ERK1/2, Rac1 and Cdc42 activations

Both GDNF and NRTN have previously been shown to regulate neurite outgrowth in various neuronal systems (Akerud et al., 1999; Wanigasekara and Keast, 2005; Yan et al., 2003). However, the involvement of GFRα1b in neuritogenesis is not known. To investigate possible morphological changes induced by the activation of the GFRα1 isoforms, the transfectants were stimulated with either GDNF or NRTN. When stimulated with GDNF or NRTN, cells expressing GFRα1a but not GFRα1b showed extensive neurite outgrowths (Fig. 3). Unexpectedly, cells expressing GFRα1b showed naïve neuroblast morphology upon ligand stimulations, comparable to cells transfected with control vector alone (Fig. 3A). These cells extended neurite-like structures when treated with retinoic acid, indicative of the potential for neurite outgrowth. GDNF and NRTN have no neuritogenic effect on control vector transfected Neuro2A cells (data not shown). Similar results were also obtained in Neuro2A cells transiently infected with retrovirus encoding GFRα1a or GFRα1b (Fig. S1), excluding the possible interference of prolonged antibiotic selection.

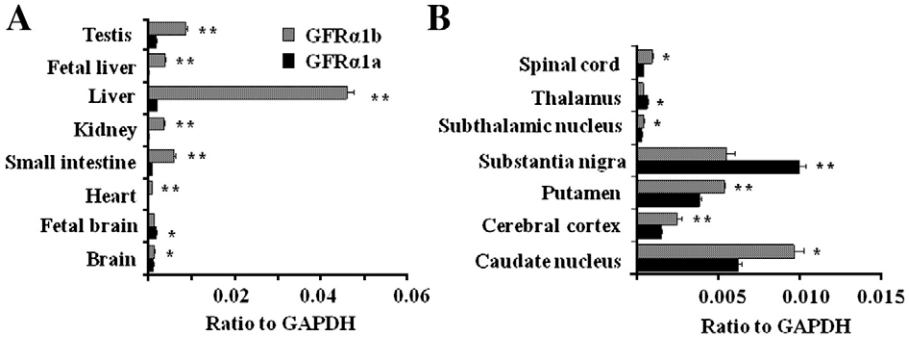

Fig. 1. Differential expressions of GFRα1 isoforms in various human tissues and central nervous system. The expression levels of GFRα1 isoforms in various human adult and fetal tissues (A) and in various regions of adult central nervous system (B) were measured. The results were expressed as mean ± S.E.M. ($n = 3$). *$p < 0.05$; **$p < 0.005$ (unpaired two-tailed *t*-test).