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Research Report

Role of fibroblast growth factor 8 in neurite outgrowth from spiral ganglion neurons in vitro ☆

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ABSTRACT

Many neurons degenerate after injuries resulting from overstimulation, drugs, genetic mutations, and aging. Although several growth factors and neurotrophins delay degeneration and promote regrowth of neural processes, the role of fibroblast growth factor 8 (FGF8) in mammalian spiral ganglion neurons (SGN) neurite outgrowth has not been examined. This study develops and uses SGN cell cultures suitable for experimental analysis, it investigates whether FGF8a and FGF8b isoforms affect the neurite outgrowth from SGN cultured in vitro. We found that both FGF8a and FGF8b promoted the outgrowth of neurites from cultured SGN. This response is mediated by FGF receptors and involves the activation of IκBα-mediated NFκB signaling pathway. These findings suggest that, besides its morphogenetic role during development, FGF8 may have trophic functions in the adult which are relevant to regeneration.

1. Introduction

Fibroblast growth factor 8 (FGF8) is a member of the FGF family. It plays a pivotal role in the patterning of the brain during embryogenesis (Crossley and Martin, 1995; Suzuki-Hirano and Shimogori, 2009). Unlike other FGFs, a relatively large number of FGF8 isoforms has been described, which are products of alternative splicing (Crossley and Martin, 1995; Olsen et al., 2006). Eight FGF8 isoforms have been found in the mouse (FGF8a–h) and four in humans (FGF8a, b, e and f). FGF8a and FGF8b have 100% sequence homology between mouse and human (Sunmonu et al., 2010). FGF8 elicits its effects by the activation of four different subtypes of tyrosine kinase FGF receptors, named FGFR1 through FGFR4 (Zhang et al., 2006). The FGF8a and FGF8b structures and their receptor-binding affinity properties have been well characterized (Olsen et al., 2006). The single residue phenylalanine 32 (F32) from the alternatively spliced N-terminal region of FGF8b confers on it a higher binding affinity than FGF8a toward the "c" isoforms of their receptors FGFR1–3 and FGFR4 (Olsen et al., 2006).

Previous studies in vitro have implicated other FGFs and neurotrophins in migration, differentiation, neurite outgrowth (Hossain et al., 2008; Hossain and Morest, 2000), survival and regeneration of primary auditory neurons (Wang and Green, 2011; Wei et al., 2007). The role of FGF8 in the nervous system, and particularly in the auditory system, has received less attention. For these reasons we have used the auditory system as a model to investigate the function of FGF8.

The cochlear spiral ganglion neurons (SGN) convey sensory information representing environmental sounds from the hair cells in the cochlea to the cochlear nucleus in the brain. SGN degenerate after injuries resulting from noise,

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drugs, genetic mutations, and aging (Bao and Ohlemiller, 2010; White et al., 2000). Since SGN are the primary cells in a pathway linked to the development and maintenance of language, learning, communication and social interaction, it would be advantageous especially to arrest this pathology and promote regrowth of SGN. Since growth factors and neurotrophins can delay degeneration of SGN and other neurons, and promote regrowth of neural processes (Gillespie and Shepherd, 2005; Klimaschewski et al., 2004; Miller et al., 2007; Sapieha et al., 2003), we investigated whether FGF8 promoted the growth of neurites from dissociated SGN cultured in vitro, and which signaling pathway might be involved. These cultures allowed accurate measurements to be made of individual neurites growing from the neurons.

In the auditory system, FGF8 is required during development for otic placode induction, pillar cell differentiation and the patterning of the organ of Corti (Jacques et al., 2007; Ladher et al., 2005; Leger and Brand, 2002; Pirvola et al., 2000). It acts by activating FGFR1, FGFR2 or FGFR3 receptors, which are expressed in the cochlea during development (Hayashi et al., 2007; Jacques et al., 2007; Leger and Brand, 2002; Oelling et al., 1995). FGF8 also functions as an axon guidance cue during CNS development (Irving et al., 2002). One could predict, therefore, that FGF8 may have a role in re-establishing synaptic connections after hearing loss (Webber and Raz, 2006). For example, FGF8 may participate in axonal regeneration by promoting regrowth of SGN processes, since it promotes neurite outgrowth in explants of chicken vestibular and auditory neurons (Fantetti and Fekete, 2012).

Based on the functions of FGF8 discussed above, we hypothesize that FGF8 may promote neurite outgrowth from mammalian SGN, a function that has not yet been investigated. The present study focuses on whether FGF8a and FGF8b isoforms could function as trophic factors in the mouse cochlea. Using dissociated mouse SGN cultured in vitro, we determined if these isoforms affect neurite outgrowth, and the possible receptors and signaling pathways involved.

2. Results

2.1. DCX staining of the neurites

To measure the length of the neurites of SGN in vitro, we used two neuronal markers. In addition to the widely used neuronal marker directed against β-tubulin III (TUJ1), we used the microtubule-associated protein doublecortin (DCX), because of its distribution at the ends of neuritic processes (Friocourt et al., 2003). We found that, although both antibodies are specific for neurons, staining both the soma and the neurites of the SGN, the anti-DCX antibody stains the neurites out to their ends, which are labeled faintly or unlabeled with the anti-TUJ1 (Fig. 1). Therefore we used anti-DCX in the subsequent experiments to visualize neurites in vitro.

As revealed by TUJ1 and DCX immunostaining (Figs. 1 and 3), we observed bipolar and multipolar SGN, which are typical of dissociated SGN (Whitlon et al., 2006) grown in culture and immature SGN (Hossain et al., 2008).

2.2. Survival of neurons with the treatments

We evaluated the effect of treatments on cell survival by counting the number of SGN per mm^2. We found no significant differences between treatments (Table 1). This result rules out the possibility that the effect of treatments on neurite outgrowth can be attributed to differences in survival as reflected in the number of neurons.

2.3. FGF8a and FGF8b effects on SGN neurite outgrowth

We tested the effects of FGF8a or FGF8b in vitro on the neurite outgrowth from primary auditory neurons. Although neither isoform had an effect on the survival of the SGN (Table 1), we found a 39.2±3.1 (mean±SE) percent increase in the length of neurites in cultures treated with 250 ng/ml of FGF8a, compared to untreated controls; at higher doses the FGF8a did not promote any further increase in the length of neurites (Fig. 2A). FGF8b promoted a 15.3±2.8 (mean±SE) percent increase of neurite outgrowth when it was present at 5 or 50 ng/ml. This stimulation was only 6.9±2.3 (mean±SE) percent when FGF8b was used at 250 ng/ml (Fig. 2B).

2.4. Effect of FGFR inhibitor on FGF8-stimulated neurite outgrowth

To determine if FGF8 receptors mediated the FGF8-stimulation of neurite outgrowth, we tested the effect of the FGF receptor inhibitor PD173074 (Bansal et al., 2003; Skaper et al., 2000). By exposing the cultures to 100 nM PD173074 and either FGF8a (250 ng/ml) or FGF8b (5 ng/ml), the FGF8-stimulated neurite outgrowth was suppressed (Fig. 2C). PD173074 did not have any additional effect on FGF8-stimulated neurite outgrowth when present at 200 nM (data not shown). In addition, the inhibitor had no discernible effect on SGN survival (Table 1) or on the morphology of the cell bodies. These findings suggest that FGF receptor activity may be required for FGF8-stimulated neurite outgrowth.

2.5. Interaction of FGF8a and FGF8b

To evaluate whether FGF8a or FGF8b isoforms have a synergistic or competitive action, we exposed the SGN cultures to both FGF8a and FGF8b at concentrations of 250 and 5 ng/ml, respectively. Despite the higher concentration of FGF8a, its effect on neurite outgrowth (Fig. 3B and E) was suppressed in the presence of FGF8b (Fig. 3D and E). In contrast, the effect of FGF8b remained the same, whether it was present alone or together with FGF8a (Fig. 3C–E). These findings are consistent with a higher affinity of FGF8b for the receptor, as reported previously (Olsen et al., 2006), and it suggests that FGF8b can competitively interfere with the action of FGF8a.

Therefore in another test, we first pre-incubated the FGF8b with the anti-FGF8b antibody, to neutralize its activity. Then we exposed the cultures to FGF8a, the combination of FGF8a and FGF8b, or the combination of FGF8a and the neutralized FGF8b. The neutralized FGF8b had no significant effect on the action of FGF8a on neurite outgrowth (Fig.3F). This suggested that FGF8a can exert its full effect on neurite outgrowth only when FGF8b is absent.