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Localization and Expression of GFRα2 Receptors in Neonatal and Adolescent Mouse Heart.

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LOCALIZATION AND EXPRESSION OF GFRα2 RECEPTORS IN NEONATAL AND ADOLESCENT MOUSE HEART

Thesis submitted in partial fulfillment of Honors

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Abstract

Neurturin (NRTN), a member of the glial cell-line derived neurotrophic factor (GDNF) family, is required in the development of parasympathetic cholinergic neurons. It signals through binding to a glycosyl-phophatidyl inositol (GPI)-linked receptor, GDNF family receptor α2 (GFRα2), which couples to Ret tyrosine kinase. Studies of NRTN knockout mice have shown that NRTN is essential for normal cholinergic innervation of the heart, but the precise role of NRTN remains unknown. For NRTN to evoke a biological response, GFRα2 must be localized to the surface of target neurons. The aim of this study was to determine the expression and localization of GFRα2 at two developmental time points in the atria of mouse hearts, postnatal day (P)1 and P18. Atria were used because of their extensive cholinergic innervation, particularly at the sinoatrial node and the right atrium. By P21, neurons are adult sized and substantial growth of cholinergic nerve fibers has occurred; therefore, it was hypothesized that GFRα2 signaling would be higher in P1 neuronal fibers than in P18 fibers. Because NRTN only activates surface receptors, and once activated, internalization occurs, we further hypothesized that GFRα2 would be cytoplasm localized when treated with NRTN. Atria were analyzed for GFRα2 expression in cholinergic neurons by immunohistochemistry with and without Triton X-100, a cell membrane permeabilizing detergent, to visualize cytoplasmic localization in one group and cell membrane localization in the other. In an additional group, excised P1 atria were cultured in petri dishes with and without NRTN (400 ng/ml, 3h) to determine if GFRα2 was internalized in response to NRTN treatment. Stained atria were viewed using a fluorescence microscope, and digital images were collected using a confocal microscopy system. Within each age group, Triton X-100

treated tissues exhibited cytoplasmic localization within ganglia; however, P1 neurons had distinct membrane staining, whereas in the P18 model, the majority of the GFRα2 was localized to the cytoplasm. NRTN-treated P1 ganglia showed a substantial decrease in membrane localization in central cell bodies and an increase in localization in perimeter cell bodies compared to the non-NRTN group. The results from this study show that GFRα2 is extensively localized to the cell membrane in P1 cholinergic neurons and is primarily localized to the cytoplasm in P18 cholinergic neurons. NRTN treatments lead to internalization of GFRα2 and may lead to a better understanding of GFRα2 trafficking. These findings suggest that GFRα2 cellular localization may be increased in periods of elevated nerve fiber growth and may serve as a regulator of responsiveness to NRTN.

1. Introduction

The parasympathetic nervous system, a branch of the autonomic nervous system, is responsible for maintaining the body in a state of rest and relaxation. It allows digestion to take place, slows heart rate, lowers blood pressure, decreases cardiac contractility force, and slows breathing. The majority of parasympathetic stimulus to the bodily organs originates in the brainstem and travels via the vagus nerve. The preganglionic vagal fibers synapse with and innervate ganglia, collections of neuron cell bodies, in the effector tissues such as the heart. These ganglia use acetylcholine as their neurotransmitter and are thus termed cholinergic neurons. The nerve fibers that extend out of these ganglia travel through the tissue to give parasympathetic innervation to the tissue.

Neurotrophic factors, which include the glial-cell line derived neurotrophic factor (GDNF) family, are crucial for the development of autonomic nerves and the innervation of target tissues such as the heart[1-4]. The GDNF family includes four members: GDNF, neurturin, artemin, and persephin[1,2]. These ligands signal through two component receptors: a glycosyl-phosphatidyl inositol (GPI)-linked receptor and Ret tyrosine kinase[1]. The GPI-linked receptor functions as a ligand binding component and Ret functions as an intracellular signaling component[1]. A GPI-linked receptor exists for each of the four GDNF family members; therefore, the four receptors are termed GDNF family receptors (GFRα). Each family member binds preferentially to one of the four receptors: GDNF to GFRα1, neurturin to GFRα2, artemin to GFRα3, and persephin to GFRα4. The GFRα receptors couple with Ret to activate internal signaling. The GDNF family of ligands have been linked to parasympathetic development in mammals[5-8].

The exact role of neurturin (NRTN) in the development of cholinergic neurons and nerve fibers in the heart remains unknown; however, it is essential for normal cholinergic innervation of the heart. In experiments with NRTN-knockout mice or GFRα2-knockout mice, major deficits of cholinergic innervation have been found in the cardiac tissue of adults[9,10]. Embryonic day (E)18 Ret-knockout mice have also shown reduced cardiac ganglion volume and die shortly after birth[9]. NRTN-knockout mice exhibited a 65% reduction of cholinergic neuronal cell bodies within cardiac ganglia and almost a 90% loss of cholinergic nerve fibers at the sinoatrial (SA) node[10]. Atrial concentrations of acetylcholine, the neurotransmitter of cholinergic neurons, were also decreased by 74% in knock-out mice. All of these factors show evidence that neurturin is required for normal development of cardiac parasympathetic innervation.

Cardiac parasympathetic neurons express Ret and GFRα2, the receptors of NRTN, in rat models from E18 through adulthood[9]. This suggests that NRTN should evoke a response from cardiac neurons at any point in this time period. The mRNAs of NTRN, GDNF, and their receptors were detected by *in situ* hybridization in various tissues, including the heart, of developing mice from embryonic day (E)12-E18[11]. In the atria, NRTN was expressed at moderate levels, and GFRα2 was also expressed at moderate levels[11]. This expression further justifies NRTN as a neurotrophic factor of cardiac parasympathetic neurons because of the extensive cholinergic innervation that is concentrated in the atria[12-14].

GFRα2 is transcribed from the short arm of chromosome 8[15], and the complete receptor is trafficked to the cell membrane where it becomes functional[16]. NRTN binds to functional GFRα2 (K_d ~ 10pM) on membranes which then complexes with the signaling

component Ret[16]. The complex is trafficked retrograde to the cell bodies with continual signaling by Ret[17]. The localization of GFRα2 receptors during development has not been studied, nor have the effects of NRTN on GFRα2 localization been studied. In order to determine the localization of the receptors at different postnatal developmental points, we examined presence and localization of receptors in P1 and P18 atria. Cholinergic innervation of the atria reaches maturity at P21, and by P18, significant growth has occurred[18]. P1 and P18 atria were visualized, in the absence of NRTN treatment, with and without Triton X-100, a membrane permeabilizing detergent, to determine the localization and intensity of expression in the cytoplasm and on the membrane. P1 atria were also evaluated to determine if NRTN treatment affected the cellular localization of GFRα2.

2. Materials and Methods

2.1 Experimental Animals

P1 and P18 C57BL/6 mice were used in this study. Animal protocols were approved by the East Tennessee State University Animal Care and Use Committee and conformed to the guidelines of the US National Institutes of Health published in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996.). Mice were deeply anesthetized using 5% isoflurane and euthanized by decapitation. The hearts were quickly removed and the atria dissected from the ventricles. The atria were fixed in a 4% paraformaldehyde solution (4°C, 24hr), washed in a solution of phosphate-buffered saline (PBS) (0.1M, pH 7.3, room temperature), and stored in PBS at 4°C until staining.

2.2 In Vitro Treatment with NRTN

Atria were placed in petri culture dishes with Dulbecco's Modified Eagle's Medium (Sigma, D5546) containing 10% fetal bovine serum (Gibco, 26140), 2 mM L-glutamine (Sigma, G7513), 100 units/mL Penicillin G, 0.1 mg/mL Streptomycin (Sigma, P0781) and NRTN (400ng/ml). The atria were cultured at 37°C in an incubator for 3hr, fixed in 4% paraformaldehyde solution (4°C, 24hr), and washed in PBS.

2.3 Immunohistochemistry

Immunohistochemistry was performed on whole atria. This was done because the goal was to visualize cytoplasmic staining separately from membrane staining. To do this whole cells were needed. If atria were sectioned prior to staining, cellular cytoplasm would be exposed and antibodies allowed to bind. Atria were blocked in a blocking solution of 5% normal donkey serum (Jackson ImmunoResearch Laboratories) and PBS for 2hr at room temperature. Atria were then treated with primary antiserum generated in goat (72hr, 4°C) in a PBS and 5% normal donkey serum solution. NRTN cultured atria were also treated with primary antiserum generated in rabbit. Primary antibodies used include goat anti-GFRα2 (1:250; R&D Systems) and rabbit anti-Nerve Growth Factor (p75) receptor (1:1000; Advanced Targeting Systems). Atria were briefly washed in PBS, blocked in blocking solution (2hr, room temperature), and treated with species-specific secondary antibodies conjugated to Alexa Fluor 488 and, in NRTN cultured atria, Alexa Fluor 555 (48hr, 4°C) (Molecular Probes). This was done in a solution of PBS and 5% Normal Donkey Serum. Secondary antibodies were used at a dilution of 1:200. After

incubation with secondary antibodies, the atria were washed extensively with PBS, frozen on specimen plates with powdered dry ice, and sectioned at 20μm thickness using a Leica CM3050S cryostat (Leica Microsystems Inc.). Sections were collected on charged slides and allowed to dry thoroughly before coverglasses were attached using Citifluor mounting medium (Ted Pella, Inc.) and sealed with clear nail polish. Triton X-100 (0.4%), a membrane permeabilizing agent, was added to blocking, primary, and secondary solutions in atria intended for cytoplasmic visualization.

2.4 Confocal Microscopy and Fluorescence Imaging

Tissue sections were viewed using an Olympus BX41 microscope (Olympus America, Inc.), and the cardiac ganglia were photographed using an Optronics MagnaFire SP charge-coupled device camera. Sections containing the SA node were also photographed. Cardiac ganglia indentified with the Olympus microscope were evaluated by laser scanning confocal microscopy with a Leica TCS SP2 confocal microscope system.

3. Results

3.1 P1 Atria Show Strong Membrane Expression in Cardiac Ganglia

Atria treated without Triton X-100 show GFRα2 membrane localization only. Localization of GFRα2 to the neuronal membrane is much more apparent in P1 atria stained without Triton X-100 than in the P18 atria (Figure 1A, 1C). The P1 ganglion shows crisp membrane localization on each of the cells within the ganglia. In contrast, the

P18 ganglion shows weak membrane signals and differentiation between cells is difficult to determine (Figure 1C).

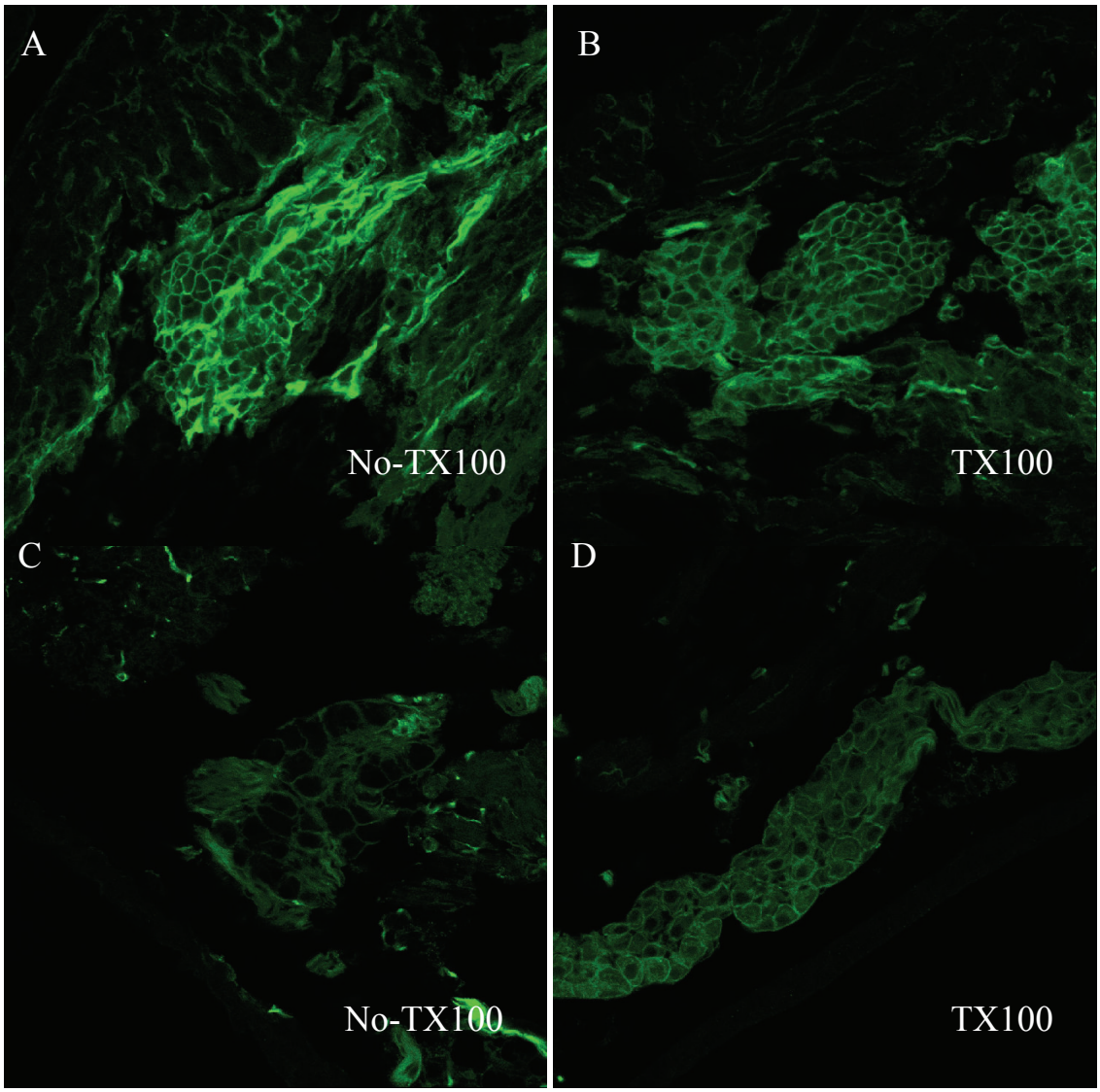

Figure 1. Staining for GFRα2 in P1 and P18 atria processed in the absence and presence of Triton X-100. P1 and P18 atria were treated with Triton X-100, a membrane permeabilizing agent, and then stained using GFRα2 primary and Alexa Fluor 488-conjugated secondary antibodies. Membrane staining was more prevalent in P1 atria than P18 atria **(A,C).** Both age groups exhibited cytoplasmic staining with the Triton X-100 treatment **(B,D).**

3.2 Cytoplasmic Expression of GFRα2 in Cardiac Ganglia and Neuronal Fibers

Triton X-100 was used to permeabilize cell membranes in order for antibodies to bind to GFRα2 in the cytoplasm. Although it is difficult to compare concentrations in the cytoplasm because of the lower volume in P1 neuronal cell bodies, differential localization can still be examined. P1 cell bodies showed cytoplasmic GFRα2 localization (Figure 1B), whereas in P18 neuronal cell bodies, GFRα2 was primarily localized to the cytoplasm, and not to the cell membrane (Figures 1C, 1D).

At the SA node region of P18 atria treated with Triton X-100, immunostaining for GFRα2 revealed a dense plexus of nerve fibers (Figure 2B). Tissue not treated with Triton X-100 exhibited staining primarily in the larger nerve fiber bundles (Figure 1A).

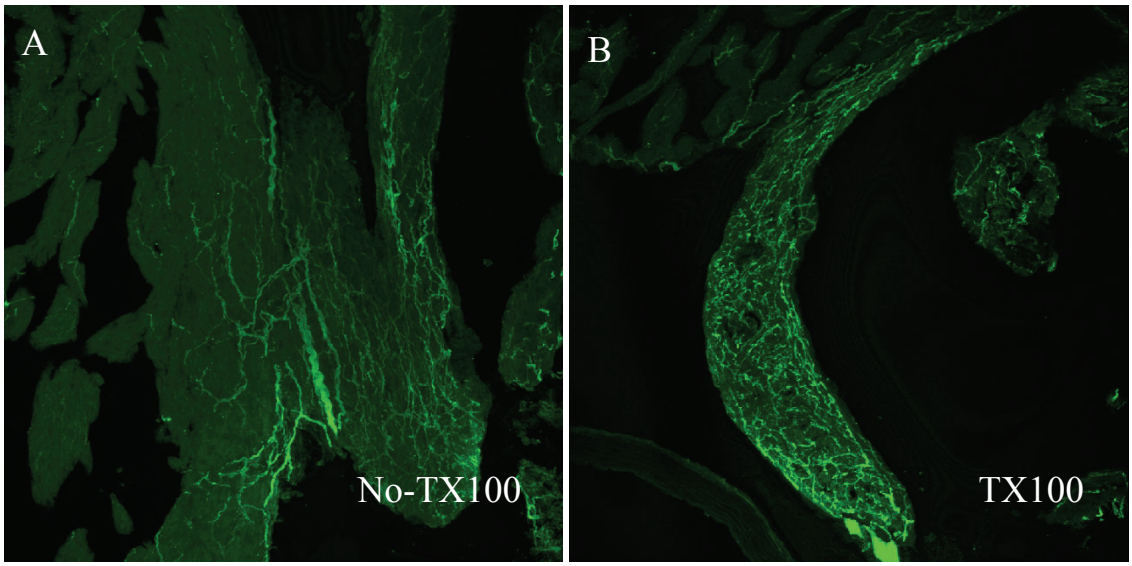

Figure 2. Staining for GFRα2 in SA node region of P18 atrial nerve fibers processed in the absence and presence of Triton X-100. P18 atria were treated with Triton X-100, a membrane permeabilizing agent, to visualize cytoplasmic expression and stained using GFRα2 antibodies and Alexa Fluor 488-conjugated secondary antibodies. At the SA node region, control atria (**A**) showed lighter and less abundant staining of neuronal fibers than the Triton X-100 atria (**B**). This suggests that more GFRα2 is inside the fibers than on the membrane.

3.3 NRTN Treated Cardiac Ganglia

Staining of cardiac ganglia after treatment with NRTN showed cell bodies on the perimeter of the ganglia having strong membrane expression of GFRα2; however, central cell bodies had minimal staining on the membranes (Figure 3A). Atria not cultured in NRTN exhibited uniform staining of membranes throughout the ganglion (Figure 3B).

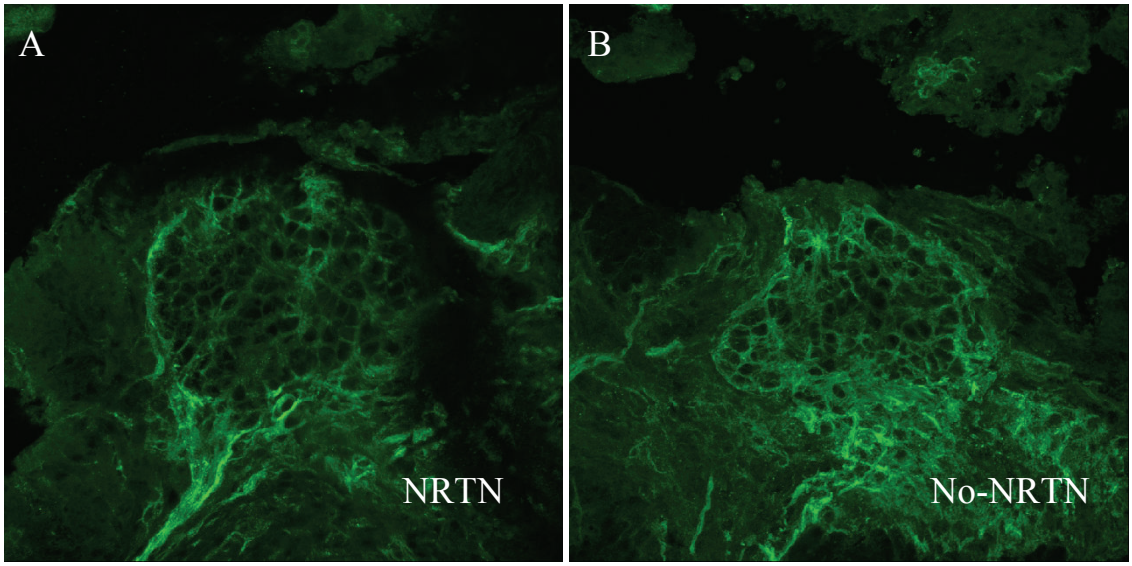

Figure 3. Staining for GFRα2 in cardiac ganglia of P18 atria treated with and without NRTN. Atria were cultured with and without NRTN (400ng/ml, 3hr) to determine effect of NRTN on GFRα2 localization. NRTN cultured tissue exhibited non-uniform staining (**A**) unlike control ganglia (**B**).

4. Discussion

P1 neuronal cell bodies exhibited much stronger GFRα2 membrane staining than the P18 neuronal cells. At P1, substantial nerve growth still needs to occur before maturity. Cell bodies are small, the volume is dominated by the nucleus, and neurite outgrowth is minimal throughout the atria. At P18, cell body size and regional nerve density in the atria are comparable to what is observed in adult atria. Cell bodies have doubled in size and neurite outgrowth is near completion[18]. Because NRTN is involved in cholinergic neural growth, it was hypothesized that the receptors would have a greater

expression on the cell membranes at P1 than at P18. P1 neuronal bodies also exhibited cytoplasmic staining which may be due to the internalization of the GFRα2 and NRTN complex. The ligand/receptor complex, upon NRTN binding, is internalized and signals as it travels toward the nucleus[17]; therefore, cytoplasmic staining would be expected. Staining could also be from reserve GFRα2 waiting to be transported to the cell surface. A method to find the quantity of internalized NRTN/GFRα2 complexes would be beneficial in determining how much of the cytoplasmic staining exists because of internalization. Culturing the tissue in a NRTN absorbing antibody would remove endogenous NRTN from solution thus giving a clearer understanding of what is happening with GFRα2. Because NRTN is not produced in the neuron, it must be secreted into the interstitial fluid to bind to the GFRα2 receptors on neuronal surfaces. The presence of an absorbing antibody would inhibit binding to GFRα2 by binding to NRTN and altering its conformation. If this were allowed to take place in culture at a high antibody concentration for a prolonged period, it would be expected that little-to-no NRTN would bind to GFRα2, and NRTN already internalized would be broken down. Beyond determining the level of cytoplasmic staining caused by NRTN internalization, this experiment could also give a better understanding of the concentration of GFRα2 receptors on the cell membrane. It could also indirectly show the effect of NRTN on GFRα2 expression, that is, whether GFRα2 transcription and expression increases in the presence of NRTN, or if there is increased trafficking of extant pools of GFRα2 to the cell membrane.

In P18 atria treated with Triton X-100, GFRα2 was primarily localized in the cytoplasm of ganglionic neurons. Because nerve growth is nearing its endpoint at P18, it

was hypothesized that NRTN activity would be decreased as compared with activity in P1 neurons, and, thus, GFRα2 expression would also be decreased throughout the cells. Cytoplasmic GFRα2 could be present due to its presence in complexes with NRTN, or it be could stored in cytoplasmic storage vesicles until stimulated to translocate to the cell surface. NRTN could act in low quantities throughout life, which would also lead to expression of GFRα2 in or on the neuron. Another possibility is that GFRα2 may act as a regulator of NRTN responsiveness, and, at this age point, GFRα2 is only expressed at low levels on the cell membrane. Confocal microscopic images of the SA node region support the localization of GFRα2 predominantly to the cytoplasm (Figure 2). Treatment of the P18 atria with a NRTN absorbing antibody may support or put to rest these hypotheses put forth to explain the high cytoplasmic staining in P18 neurons.

Atria cultured in NRTN displayed non-uniform staining throughout the ganglia (Figure 3A). The marked decrease in membrane staining of the NRTN cultured ganglia (Figure 3A) compared to the ganglia cultured without NRTN (Figure 3B) provides supporting evidence for the internalization of GFRα2 by NRTN. The 3hr treatment at levels well above the NRTN saturation point (100ng/ml), may have been long enough to allow GFRα2 in perimeter cells of the ganglia to bind NRTN and signal cytoplasmic receptors to traffic to the cell membrane. Cells in the interior of the ganglia probably had a shorter exposure time to the NRTN, due to the binding in perimeter cells and the longer penetration time. The 3hr treatment may have been just long enough for these cells to come in contact with NRTN and for GFRα2 to become bound and internalized. The kinetics of GFRα2 trafficking has not been studied to date; therefore, the true events occurring are left to speculation. One prospect of determining trafficking would be

through tagging NRTN molecules with a radioactive tag and tracing the binding and cytoplasmic paths of the molecules over the course of time. It has been found using a similar method that GDNF, a growth factor in the same family as NRTN, binds GFRα2 and internalizes ~30-40% of total cell surface complexes within 2min in the absence of Ret[19]. Much could be learned from this experiment including GFRα2 lifespan, rate and time of NRTN internalization, complex degradation or recycling, and time to traffic to the cell membrane.

The findings in these experiments set the stage for future research that may shed light on the interactions of NRTN and GFRα2 and, possibly, may lead to elucidation of the role of NRTN in cholinergic growth. These experiments looked strictly at the localization of GFRα2 in the membrane and the cytoplasm. The findings could be supplemented with quantitative data comparing the amount of GFRα2 localized to each area. While determining cytoplasmic localization between P1 and P18 neurons would be difficult due to fiber growth differences and cytoplasmic volume differences in the age points, cell membrane localization could be easily quantified by measuring mean staining intensity differences in the cell membranes. This intensity measure could be used to compare P1 ganglia to P18 ganglia as well as NRTN treated ganglia to untreated ganglia. Intensity of perimeter cell membranes in NRTN cultured ganglia could be compared to perimeter cell membranes in non-NRTN cultured ganglia. Intensity could also be compared between the two groups for the central neuronal bodies. Measures of the difference between perimeter and central neuronal bodies within the same ganglion could also be compared. The data collected would be useful in getting a concrete difference between treatments instead of relying on visual differences.

5. Conclusions

The NRTN receptor, GFRα2, is membrane localized in P1 neuron cell bodies with cytoplasmic concentrations that may be a result of trafficking back and forth from the cell membrane. Part of the cytoplasmic staining may also be newly synthesized GFRα2 that is being trafficked to the cell membrane or waiting as reserve stores. In P18 tissue, GFRα2 is primarily localized to the cytoplasm and is minimally localized to the cell membrane in neuronal cell bodies. Treatment of P1 atria with a high concentration of NRTN for 3hr led to non-uniform staining throughout ganglia with perimeter cell bodies seemingly having higher concentrations of GFRα2 at the cell membrane and interior cell bodies have little GFRα2 localization on cell membranes. These findings suggest that GFRα2 is internalized and resupplied to the membrane of neurons at the perimeter of the ganglia and that primarily internalization has occurred for neurons located at the interior.

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