Acute Application of NT-3 on Neonatal Rat Spinal Cords

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ABSTRACT

Neuronal survival, growth and differentiation in the developing brain and spinal cord are dependant upon the presence of specific growth factors called neurotrophins. These include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 and function to regulate neuronal survival and differentiation during the development of the vertebrate nervous system by binding with high affinity to the tyrosine kinase (*trk)* family of receptors, which includes *trkA*, *trkB* and *trkC*. Neurotrophins can also bind to another receptor type named the $p75^{NTR}$ receptor of the tumor necrosis family, although binding affinity is rather low. Signaling from this receptor most commonly results in programmed cell death.

Neurotrophins may activate *trk* and $p75^{NTR}$ receptors individually or simultaneously via complexes known as heterocomplexes. The reason for study of these complexes involves their unique ability to discontinue the apoptotic signaling of $p75^{NTR}$ [1,2]. Coupled with the knowledge that *trkC* interacts exclusively with NT-3, this study aimed at determining whether acute application of NT-3 would cause an upregulation of heterocomplexes. This would serve as a mechanism to prevent $p75^{NTR}$ induced cell death. This is of particular importance in circumstances such as spinal cord injury in which $p75^{NTR}$ expression is drastically upregulated.

It was found that there were no observable differences in the amount of heterocomplex expression between acutely treated neonatal rat spinal cords and control cords, however this project yielded other unexpected yet interesting results. It was noted that labeled heterocomplex tracts possessed an abnormally high number of stained nuclei indicating that these tracts may be highly concentrated on radial glial cells since these are known to serve as guide wires to young, migrating cells.

Keywords: neurotrophin, tyrosine kinase, $p75^{NTR}$, dimerization, DRG, SCI

INTRODUCTION

Implications in Spinal Cord Injury and Disease

In early development, neurons that fail to reach their target are eliminated through the $p75^{NTR}$-induced apoptotic program. In adults however, $p75^{NTR}$ expression levels in nervous tissue are much lower thus most neurotrophic action in adults is related to neuronal survival. An important exception occurs upon central nervous system trauma in which $p75^{NTR}$ expression levels dramatically increase many cell types including motor neurons[3] and corticospinal neurons[4] in rodent experiments.

Neurotrophin Actions

NT-3 is of particular importance as it is required for establishment of sensory axon projections as evidenced by the severe movement disorders portrayed by NT-3 and *trkC* deficient mice[5,6,7]. NT-3 application has been shown to maintain and even restore sensory-motor synaptic function in neonatal rats[8] as well as increase EPSP amplitude in rat motor neurons[9]. Antibody blocking of *trkC* yields decreases in EPSP amplitude. NT3 application, either chronically[10] or acutely[11] affects sensory input to motor neurons thus proving its importance in the larger picture of neural networks.

NT-3 supports survival only when applied to cell bodies while it promotes outgrowth when localized at axons[12]. In dorsal root ganglion (DRG), both NT-3 and *trkC* are required for cell survival at an earlier stage than NGF and *trkA, which play a role in survival later in life*. *TrkC* is expressed throughout sympathetic ganglia and DRG early, but are reduced in number and location as *trkA* is turned on.

Heterocomplexes

As previously stated, when co-expressed, the two receptors form a binding complex that provides increased specificity and affinity for neurotrophin binding and enhanced trophic effects via strengthened *trk* signaling which with NT-3 yields increased survival and neurite outgrowth[13].

The ligand-induced receptor dimerization model coined by Schlessinger has stood as the primary model explaining heterocomplex formation[14, 15]. This model states that receptors are initially present as monomers in the plasma membrane and only dimerize upon binding of a ligand such as NT-3.

However, recent work has offered evidence indicating that this may not be the case. The ligand-induced model assumes cooperative binding occurs between the first receptor binding the ligand followed by recruitment of the second. Recent findings have instead indicated that negative cooperation is more likely, although specific experiments using NT-3 have not been published[16]. This new theory works under the assumption that a significant proportion of receptors should already be present in preformed but inactive dimers at the plasma membrane. A mechanism explaining how neurotrophin heterocomplexes or homocomplexes (homodimers) are "chosen" to be assembled remains unknown.

Recently, the idea that ligand binding drives the association of such complexes has also encountered difficulties. Co-immunoprecipitation studies have revealed that other similar neurotrophin receptors (GDNF and Ret protein) depend upon the presence of the neurotrophin GDNF in order to form heterocomplexes[17]. However since preassembled receptor complexes are unlikely to survive dissolution of the plasma membrane from detergents, co-immunoprecipitation studies may be misleading.

Many important questions remain such as how a preformed heterodimer remains inactive and what causes it to become activated. This study aims to add to the current knowledge base regarding how neurotrophin receptors react to ligands and how heterocomplexes are activated and assemble through the use of fluorescent tags and confocal microscopy.

Radial Glia

Even though neuronal development was not the intent of this study, the collected data offered conclusions regarding a potential role of neurotrophin receptors in radial glial cells. One main function of these cells early in development is to serve as scaffolding for which neural and glial cells are able to migrate from their site of birth to their eventual site of action. It has been strongly suggested that after these cells have carried out their developmental functions, they then differentiate into a variety of different cell types including astrocytes, oligodendrocytes and neurons[18]. Radial glia have also been shown to produce neuronal and non-neuronal cells from clones of a single cell[19]. Ultimately, the mechanism by which differentiation occurs is complex and the current knowledge base is incomplete, thus it remains as a current focus of research.

METHODS

Tissue Preparation and Neurotrophin Treatment

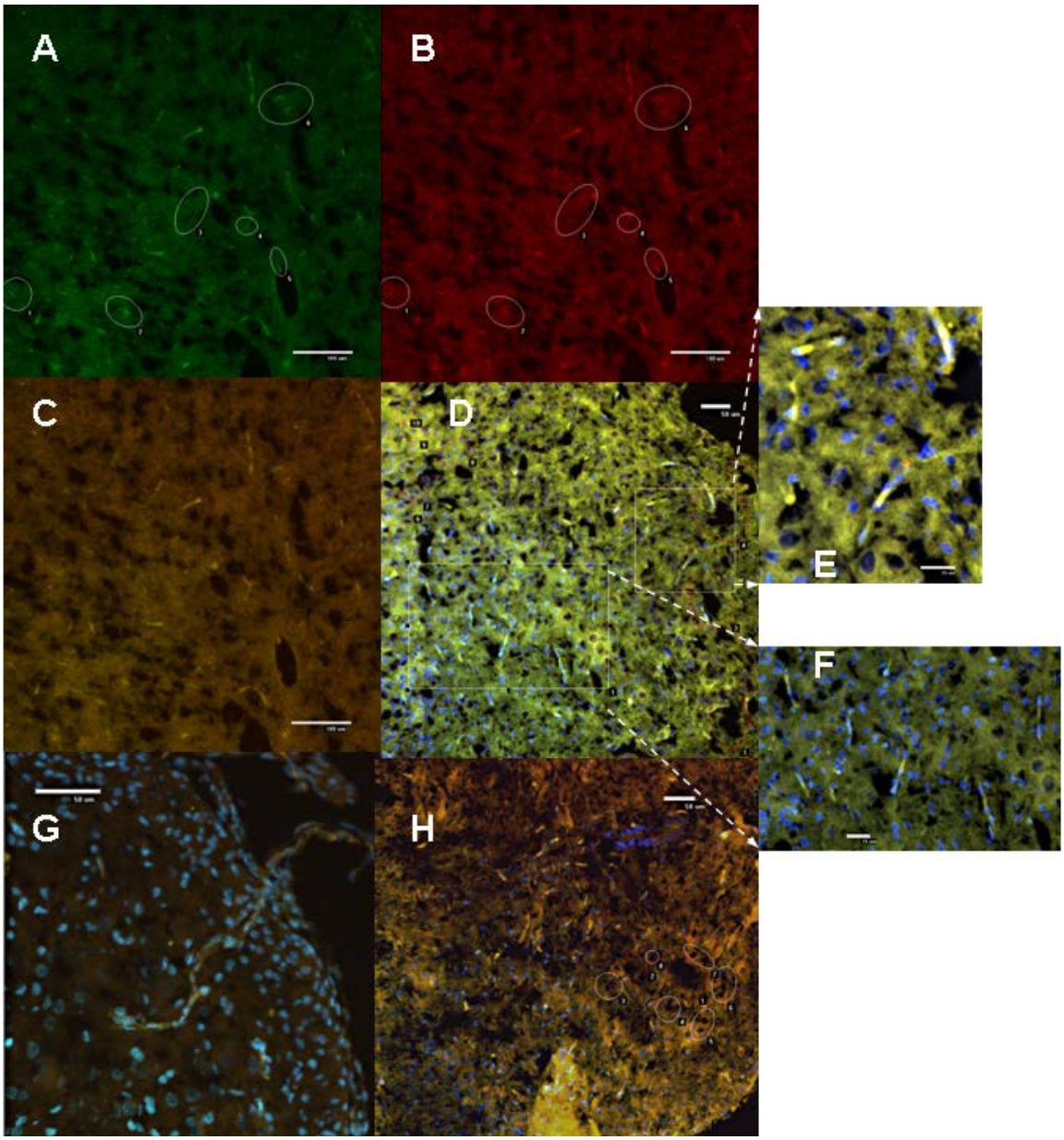
Neonatal rat pups (2-5 days) were anesthetized (using ice) and sacrificed and the spinal cords were dissected out and maintained in a CO_2/O_2 infused artificial saline (10˚C). Immediately after dissection, treated cords were placed in high NT-3 concentration (2.0 µM) solution and control cords in artificial CSF saline for 20 minutes at 10˚C. The cords were then fixed (4.0% paraformaldehyde) for 48 hours and dehydrated (30% sucrose) for 48 hours. Cords were placed in an alkane refrigerant and semifrozen in liquid nitrogen (2 minutes to remove air bubbles) then flash frozen (12 seconds). The lumbar sections of the cords were cryosectioned at 14ºC and prepared for immunofluorescence.

Fluorescence

All tissue samples were blocked for nonspecific background (10% bovine serum in PBS) for 1 hour. p75 was identified using rabbit anti-rat IgG primary antibody (1:500, Cell Signaling Technology, inc.) in 0.1% BSA in PBS and goat anti-rabbit secondary Alexa Fluor 488 (1:100) with goat block (1:50). *trkC* was identified using mouse anti-rat IgG primary antibody (1:1500, Santa Cruz Biotechnology) in 0.1% BSA in PBS and goat anti-mouse secondary Texas Red (1:500) with goat block (1:50). The secondary antibodies used with *trkC* and $p75^{NTR}$ also included nuclei-labeling dapi (1:1000). Radial glia were attempted to be identified using IgM rabbit anti-rat 3CB2 primary antibody (1:100, Hybridoma Bank) in 0.1% BSA in PBS and goat anti-mouse IgM secondary Alexa Fluor 350 (1:500, Invitrogen) with goat block (1:50). No 3CB2 fluorescence was observed using the above procedure. Confocal images were obtained using Nikon Eclipse 800 and Images were enhanced using Nikon confocal C-1 software and Adobe Photoshop.

RESULTS

Immunolabeling comparisons of $p75^{NTR}$ and *trkC* in non-merged images show significant overlap between the two receptors; however slight variances are present. Merged images comparing NT-3 treated cords with control cords do not show any noticeable differences between the expression levels of *trkC*-$p75^{NTR}$ heterocomplexes. Merged images of NT-3 treated spinal cord sections indicate a deficit of immunolabeling in suspected motor neuron pools the surrounding glia. A large number of dapi-stained nuclei are located directly along the stained heterocomplex tracts.



(A) NT-3 treated (2.0µM) rat spinal cord section (12µm thickness) dorsal to the central canal area labeling $p75^{NTR}$ tracts (white) over background tissue (green) and **(B)** *trk*C tracts (white) over background tissue (red). **(C)** NT-3 treated (2.0µM) rat spinal cord section (12µm thickness) and **(D)** control spinal cord section (6µm thickness).

Photo D is of ventral horn showing merged images of *trk*C and $p75^{NTR}$ (tracts appear white, background tissue yellow) and dapi-stained nuclei (blue) identifying absence of nuclei stain in larger gaps believed to be motor neurons (red rings). Also observable presence of nuclei stain in smaller gaps believed to be interneurons and/or glial cells (pink rings). **(E)** and **(F)** Magnified sections from D illustrating multiple dapi-stained nuclei located along *trk*C-$p75^{NTR}$ tracts. (**G)** NT-3 treated (2.0µM) rat spinal cord section (6µm thickness) ventral horn showing merge of *trk*C and $p75^{NTR}$ (tracts appear white, background tissue yellow) and dapi-stained nuclei (blue) illustrating multiple dapi-stained nuclei located along *trk*C-$p75^{NTR}$ tracts. **(H)** NT-3 treated (2.0µM) rat spinal cord section (6µm thickness) of ventral horn showing merged images of *trk*C and p75 (tracts appear white, background tissue golden) and dapi-stained nuclei (blue) identifying a lack nuclei labeling in suggested motor neuron pools (red rings) and surrounding glial cells (white rings).

DISCUSSION

It is assumed that the overlapping $p75^{NTR}$ and *trkC* immunolabeling represents heterocomplexes since the chances of these receptors being lined up vertically in a 6µm section without any interaction is extremely small. There didn't seem to be any noticeable difference in expression of these assumed heterocomplexes between NT-3 treated and untreated cords. However, no microscope features or other forms of light-detecting technology were able to be used in this determination since fluorescent emission is very sensitive to ambient light. Any slight deviation in labeling or photograph collection could impact the amount of fluorochrome emission between samples. The apparent lack of NT-3 application in heterocomplex formation is further evidence against the ligand-induced model of heterocomplex activation.

The observation that immunolabels don't seem to be uptaken at glial or neuronal cell somas is an interesting one. It is possible that since the tissue was alive up to the point of fixation, the cell somas possess a less permeable membrane compared to the growth cones at the end of the growing axons. During early development, neurite outgrowth is incredibly important thus the immunolabels may be better able to penetrate the cell membrane where it is more porous (such as at the growth cones). The high concentration of *trkC*-$p75^{NTR}$ heterocomplexes on growing neurites makes sense since these have a very high affinity for NT-3 which promotes growth upon binding to axon-located receptors.

The observation of uncharacteristic numbers of dapi-stained nuclei located directly along heterocomplex tracts lead to the presumption that these tracts are represented upon radial glial axons. The known role of radial glia to serve as scaffolding or guide wires for other cells to migrate to their location of function supports this idea since each nucleus could very well represent a migrating cell. Attempts to immunolabel radial glia in these rat spinal cord sections with a 3CB2 specific antibody were unsuccessful, thus this theory remains with little supporting evidence.

CONCLUDING REMARKS

Receptors for neurotrophins consist of dimers often between 2 different receptor types. The details of how these assemble, activate, and how/ if they deactivate are all areas undergoing research. Further investigation may require the use of fluorescence resonance energy transfer (FRET) to conclude protein-protein interaction of *trk*C and p75 heterocomplexes. Also, the use of electron-dense tags in varying sizes with electron microscopy to visualize receptor complex conformation at high resolution will allow for evaluation of the specific changes that the *trkC*-p75 complex undergoes activation.

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