Effect of NRIF on p75 Expression

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BRIEF. NRIF may be a transcriptional repressor of p75 expression.

ABSTRACT. During nervous system development an excess of neurons is generated; these compete for a limited quantity of trophic factors. Through programmed cell death, or apoptosis, approximately 50% of all neurons generated in mammals die. Understanding neuronal death regulation is essential for developing therapeutic strategies to treat neuropathologies involving abnormal apoptosis such as Alzheimer's disease. One group of trophic factors, neurotrophins, regulates apoptosis, in part, by binding to the p75 neurotrophin receptor, which induces apoptosis through association with a DNA binding protein, NRIF. It is hypothesized that NRIF is also a transcriptional repressor of p75, acting in a negative feedback loop. Using a Luciferase Reporter Assay in HEK293 cells transfected with different levels of a Gal4-NRIFconstruct, NRIF was found to repress transcription in a dose-dependent manner. To determine if NRIF regulates p75 expression *in vivo,* wild type and NRIF knockout mouse brains were sectioned and immuno-stained for p75. Wild type mice have NRIF present and NRIF knockout mice are lacking the NRIF protein. Quantification of p75 staining revealed that mice lacking NRIF have increased p75 expression, specifically in the hypothalamus and internal capsule regions. These results support the hypothesis that NRIF acts as a transcriptional repressor of p75.

INTRODUCTION.

The nervous system controls both involuntary and voluntary actions, such as movement, breathing, and thought. During nervous system development, an excess of neurons compete for a limited supply of trophic factors [1]. Trophic factors are proteins required for maintaining neurons, which function by signaling for a cell's survival or death. Through apoptotic regulation by trophic factors, and other factors, about 50% of neurons in the mammalian nervous system die either before birth or shortly thereafter. Apoptosis, or programmed cell death, is the basic process of a cell dying in response to a specific signal.

One group of trophic factors, neurotrophins, regulates apoptosis, in part, by binding to the p75 neurotrophin receptor, which associates with the DNA binding protein, neurotrophin receptor interacting factor (NRIF). The p75 neurotrophin receptor can signal for either cell survival or cell death through binding neurotrophins, such as, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), Neurotrophin 3 (NT3), and Neurotrophin 4 (NT4) [2].

Depending on the cell type and the specific neurotrophin, ligand binding to p75 causes cleavage at the extracellular domain by the tumor necrosis factor-α converting enzyme (TACE), followed by release of the intracellular domain. P75 cleavage promotes NRIF translocation into the nucleus causing gene transcription leading to cell death (Figure 1). NRIF is believed to impact apoptotic signaling by acting as a transcriptional regulator [3], but the regulation of this signaling pathway is poorly understood.

NRIF regulates gene expression [3] and is thought to be a transcriptional repressor based on its predicted structure. An earlier study showed the p75 receptor is not present under normal conditions in the hippocampus of wild type mice, but mice lacking NRIF (NRIF knockout mice) appeared to have p75 expression in this region [4]. However, this observation was not quantified, and other portions of the brain were not examined. NRIF knockout mice were genotyped beforehand to show there was no NRIF DNA present following deletion of the gene. The focus of the work described here is to define and quantify the expression patterns of p75 in NRIF knockout mice throughout the brain, including the cerebellum. P75 expression has never been analyzed in NRIF knockout mice.

P75 is expressed on cholinergic neurons in the basal forebrain, and are among the first to die in neurodegenerative disorders such as Alzheimer's disease,. Some reports have suggested that p75 may contribute to the neuronal death associated with the disease. P75 is up-regulated in many injuries to the nervous system and in many diseases such as Alzheimer's disease and Parkinson's disease [5]. Understanding how p75 induces death is important not only to better understand normal development, but also to design therapeutic strategies for neurodegenerative conditions.

In the brain, p75 is normally found in the basal forebrain, but is also present in the hypothalamus and around the internal capsule region [6]. Neurons in the basal forebrain are critical for memory formation and injury to this region often results in confusion and/or amnesia. The neurons in the hypothalamus are involved in regulating release of hormones from the pituitary as well as controlling feeding behavior and sleep. This study will test the hypothesis that NRIF suppresses p75 expression, which could affect the survival of these neuron populations and the behaviors they control.

MATERIALS AND METHODS.

In vitro.

Cell Culture.

Human embryonic kidney cells (HEK293-T cells) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 0.1% penicillin-streptomycin at 37C in 5% CO_2.

Transfection.

HEK293-T cells were transfected using the Calcium Phosphate method [7] with a total of 6 μg of DNA. A constant load of a Gal4-Luciferase reporter construct (2 μg) and a varying concentration of a Gal4-NRIF construct (low 1 μg, medium 2 μg, high 4 μg) were added, and renilla (1 μg) was included to assess transfection efficiency. A pcDNA construct was used to balance the total DNA load to 6μg. The zinc finger region of NRIF, the region typically associated with DNA binding, was replaced by the Gal4 DNA binding domain. The Gal4 DNA binding domain of the construct is from a bacterial protein that binds to the Gal4-luciferase promoter, which is upstream of luciferase construct, regulating luciferase expression.

Luciferase reporter assay.

A Dual-Luciferase Reporter Assay was performed to see how NRIF affects transcription (Promega). By transfecting cells with a plasmid containing the luciferase gene behind a promoter for the transcription factor Gal4, then expressing NRIF fused to the Gal4 DNA binding domain, the effect of NRIF on luciferase was quantified by adding luciferin to cell extracts and measuring light production. A change in the intensity of the glow from the luciferase correlated to increased transcription. If NRIF inhibits transcription, then higher concentrations of NRIF would lead to less luciferase, resulting in less light produced.

Western blot for NRIF expression.

Transfected cells were lysed to detect NRIF transfection efficiency using Western blot analysis. HEK293-T cell lysates were separated on a 10% acrylamide gel, and incubated in NRIF antibody (1:1000). Protein expression was visualized using enhanced chemiluminescence and film.

Statistical Analysis.

For the luciferase assay, a one-way ANOVA was run with a Tukey's post-hoc analysis. The data from the immuhistochemical quantification was subjected

to an unpaired student's t-test to calculate the significance of the results. Significant data was shown with $p \leq 0.05$. These analyses were done using Prism (GraphPad Software).

In vivo.

Sectioning and immunohistochemistry (analysis of p75 in cells).

6-8 week old male NRIF knockout and wild type mice were anesthetized with isofluorane and intracardially perfused with 4% paraformaldehyde. Brains were removed and fixed overnight in 4% paraformaldehyde before being transferred to 30% sucrose. Cerebrums were coronally sectioned (45 μm) using a cryostat and collected in one-in-five serial collections of tissue (i.e., every serial section was 225 μm apart). Cerebella were sectioned in one-in-four serial collections of tissue (i.e., every serial section was 180 μm apart).

Sections were stained using a rabbit anti-p75 primary antibody (1:1000), which was then detected by a donkey anti-rabbit biotinylated secondary antibody (1:1000), followed by horseradish peroxidase-conjugated streptavidin (1:1600), which is specific for the biotin tag on the secondary antibody.

The tissue was submerged in a 3,3'-diamniobenzidine (DAB) solution activated by hydrogen peroxide to detect p75 positive staining. After optimal staining was obtained, the reaction was stopped using multiple rinses of TBS (Tris-buffered saline, pH 7.5). The tissue was mounted onto slides and cover-slipped. GIMP and ImageJ were used to compile and count p75 positive cells present in each region.

RESULTS.

In vitro.

HEK293-T cells were transfected with Gal4-Luciferase and varying amounts of the Gal4-NRIF constructs. The transfected cells were then lysed and the lysates were run on a Western blot to verify that we had established transfected cells producing low, medium, and high concentrations of NRIF. An untransfected (UT) sample was used as a control to show no NRIF presence in HEK293-T cells. A Gal4-Luciferase construct was also used as a control, and was also run to show that there was no NRIF present without the Gal4-NRIF construct. Neither of these constructs showed NRIF expression because there was no NRIF present. The low Gal4-NRIF sample showed the lowest concentration of NRIF and the high Gal4-NRIF sample showed the highest concentration of NRIF. This data demonstrates that NRIF was effectively expressed in an increasing manner in the transfected cells.

Luciferase assays were also performed using cell lysates from the transfected HEK293-T cells. Fusion of the Gal4 binding domain to NRIF allowed measurement of how NRIF affects transcription. Untransfected cells and those singly transfected with Gal4-NRIF or Gal4-Luciferase were used as controls for background luminescence. The untransfected and Gal4-NRIF only samples showed no luminescence since they had no luciferase. The Gal4-Luciferase construct showed low luciferase expression. The luciferase assay also showed that there was an inverse relationship between NRIF expression and luciferase expression. Cells expressing the lowest amount of NRIF showed the highest luciferase expression, and the cells with the highest levels of NRIF showed the lowest luciferase expression. One-way ANOVA indicated overall significance, indicating that the levels of luciferase were significantly changing ($p=0.0473$), although a post hoc test failed to indicate significance between the individual groups (Figure 2A).

In vivo.

Analysis of p75 positive cells between NRIF knockout and wild type tissue sections demonstrated that NRIF knockout mice have a slightly higher overall p75 expression compared to the wild type, although the difference was not statistically significant (Figure 2B). The overall distribution of p75 positive cells in the NRIF knockout mice was similar to the wild type, indicating that deletion of NRIF did not result in the appearance of p75 expressing cells in a totally unique region, including the hippocampus (Table 1). This was somewhat different from the previous observation of p75 expression in the hippocampus of NRIF knockout mice, while being absent in wild type [4].

While overall p75 expression was not significantly different between the NRIF knockout and wild type, region-specific analysis showed greater expression in the hypothalamus, regions around the internal capsule, and the cerebellum between the NRIF knockout and wild type mice (Figures 2C, D and 3A, C), but there was no difference in the count of p75 positive cells in the basal forebrain region (Figures 2C and 3B). Subregions within the hypothalamus, around the internal capsule, and within the cerebellum were also compared between NRIF knockout and wild type brains (Table 1). The difference in p75 expression in the hypothalamus and regions around the internal capsule of NRIF knockout mice compared to wild type mice was statistically significant with a t-test showing $p= 0.0157$(Figure 2C). There was also an increase in p75 expression in the NRIF knockout mice compared to the wild type in the cerebellum, but this increase did not reach significance (Figure 3).

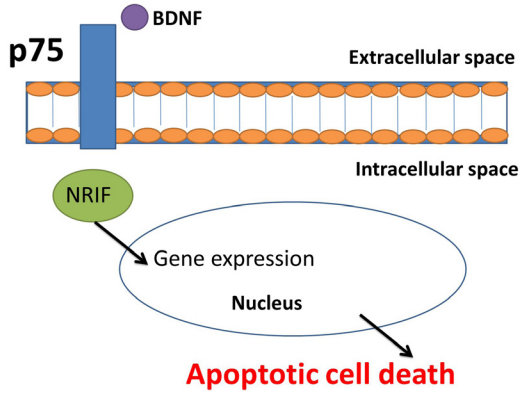

Figure 1. When a pro-death neurotrophin binds to p75 a signaling pathway is initiated. Binding causes neurotrophin receptor interacting factor, NRIF, to be transported into the nucleus where it is thought to alter gene transcription leading to cell death. NRIF is essential for apoptotic signaling.

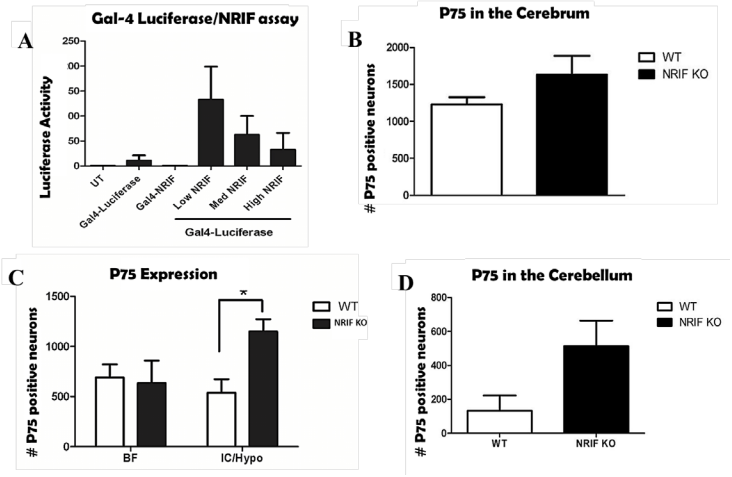

Figure 2. (A) There is an inverse relationship between NRIF and luciferase activity. Increasing NRIF expression lowers Luciferase expression. One-way ANOVA indicated overall significance p=0.0473. Post hoc test failed to indicate significance between groups. N=2. (B) NRIF knockout (KO) mice have a slightly higher p75 expression in the brain than their wild type (WT) counterparts, but did not reach statistical significance. N=4, WT; N=3, KO. (C) Students t-test revealed NRIF knockout mice have a significant increase in p75 positive cells, (* p=0.0157) compared to controls, specifically in the hypothalamus and internal capsule regions (IC/Hypo), but not in the basal forebrain (BF). N=4, WT; N=3, KO. (D) NRIF knockout mice show an increase in p75 cells, but this difference did not reach statistical significance. N=2, WT; N=6, KO.

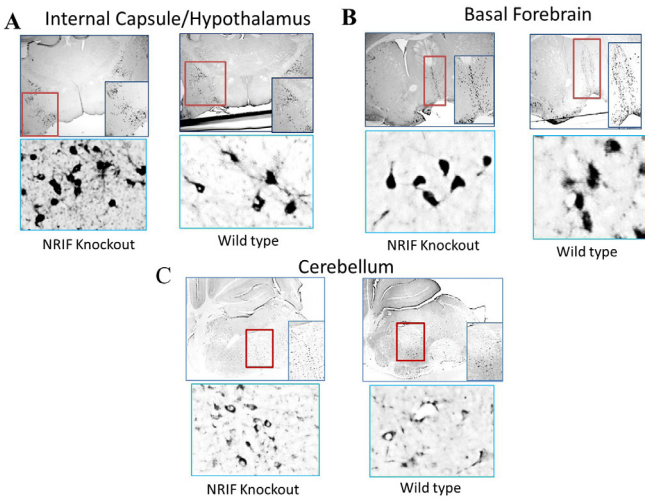

Figure 3. (A) NRIF knockout mice have a significant increase of p75 expression in the internal capsule and hypothalamus regions. Note the higher number of neurons in KO mice compared to wild type. (B) NRIF knockout and wild type mice have no change in p75 positive cells in the basal forebrain region. (C) NRIF knockout mice have an increase of p75 expression in the cerebellum region, but this increase did not reach significance. Low magnification images are 2.5x, insets are 10x, and high magnification images are 40x.

Table 1. Brain regions with p75 positive cells are compared between NRIF knockout and wild type brains. (++) shows the regions with an increase in p75 expression.

Region	*WT*	*KO*
Medial Septal Nucleus	+	+
Vertical Diagonal Band	+	+
Horizontal Diagonal Band	+	+
Substantia Innominata	+	++
Infralimbic Cortex	+	+
Dorsal Peduncular Cortex	+	+
Ventral Tenia Tecta	+	+
Medial Forebrain Bundle	+	+
Ventral Pallidum	+	+
Piriform Cortex	+	++
Magnocellular Preoptic Nucleus	+	+
Striatum	+	+
Lateral Globus Pallidus	+	+
Interstitial Nucleus of Posterior Limb of Anterior Commissure	+	++
Optic Tract	+	++
Lateral Hypothalamic Area	+	++
Medial Tuberal Nucleus	+	++
Third Ventricle	+	++
Posterior Hypothalamic Area	+	+
Magnocellular Nucleus of Lateral Hypothalamus	+	+
Medial Globus Pallidus	+	+
Internal Capsule	+	+
Ventromedial Hypothalamic Nucleus	+	++
Gigantocellular Nucleus	+	++
Dorsal Acoustic Stria	+	+
Raphe Obscurus Nucleus	+	++
Medial Longitudinal Fasciculus	+	++
Posterodorsal Tegmental Nucleus	+	++
Primary Fissure	++	+

DISCUSSION.

The data presented above indicate that NRIF acts as a transcriptional repressor, and that NRIF knockout mice exhibit increased p75 expression in the brain. At present, NRIF is only known to mediate apoptotic signaling [1]; however, recent findings indicate that NRIF can regulate the expression of other genes not directly related to cell death [3]. The findings presented here are consistent with NRIF repressing p75 expression; however, since NRIF can mediate apoptosis, it is possible that the extra cells present in the NRIF knockout mice are cells that did not die because of NRIF absence. A similar increase in certain populations of neurons has been reported in p75 knockout mice [8]. To determine if there is less cell death in brain areas with more p75 positive cells, an analysis of developmental cell death should be done. In addition, to determine if p75 is repressed by NRIF, neurons in culture could be transfected with NRIF to see if p75 expression decreases.

Previous research has shown that in the hippocampi of NRIF knockout mice there is an appearance of p75 expression where none is found in wild type counterparts [4]. The data shown above shows that there were no p75 positive cells in the hippocampus, but instead show an increase in p75 positive cells in the hypothalamus, around the internal capsule and cerebellum. It is unclear why no p75 was detected, however, it could be due to weak expression in that region relative to other regions, like the hypothalamus, which would give the impression that there was not staining. It is also possible that the antibody used in the previous study was more sensitive and could better detect p75.

The results demonstrate that NRIF can repress transcription. The Gal4-Luciferase construct showed luciferase expression that was low, as would be predicted, but it was even lower than the sample with a high amount of NRIF, which was unexpected and will need further investigation. Luciferase assay results showed that there was an inverse relationship between NRIF expression and luciferase production (Figure 2). With higher amounts of NRIF there is a lower amount of luciferase expression.

Cell counts show that there is a significant increase in p75 expression in the hypothalamus and regions around the internal capsule (Figure 2C and Figure 3A), a non-significant increase in p75 expression of NRIF knockout mice in the cerebellum (Figure 2D and 3C), and no increase in the basal forebrain region (Figure 2C and Figure 3B). It is possible that the increase in expression in the cerebellum was not significant because of variability in the small sample set that was used. There is also a non-significant increase of p75 expression in the cerebrum overall between NRIF knockout and wild type mice (Figure 2B). The basal forebrain is known to be an area of high p75 expression, specifically in cholinergic neurons [8]. The number of cholinergic neurons in p75 knockout mice is increased relative to wild type mice [8]; however, the difference was small (less than 20%). It is possible that further analysis of more animals would reveal a significant difference. Alternatively, deletion of NRIF may not protect this population of neurons from normal apoptosis.

The increased number of p75 positive cells in the hypothalamus is interesting since this is an area of the brain located in the cerebrum that controls pituitary hormones and behavioral effects. The basal forebrain, also located in the cerebrum, is involved in memory processes. The cerebellum influences motor and non-motor behavior in areas such as attention, working memory, learning, and addiction. Understanding the role of NRIF, both in its effect on p75 expression and its role in the p75-mediated signaling pathway, will aid in understanding the regulation of apoptosis which can then help in developing therapeutic strategies for neuropathologies involving abnormal apoptosis such as Alzheimer's disease and Parkinson's disease as well as lead to increased understanding of the mechanisms underlying normal mammalian neural development and function.

CONCLUSION.

The research presented above shows an analysis of the p75 neurotrophin receptor in the adult brain to see how p75 expression is affected by NRIF deletion. This could eventually lead to an understanding of how the brain is affected by p75-mediated apoptosis overall and help develop strategies for treatment of neu-

ropathologies involving abnormal apoptosis. However, more research needs to be done to fully understand neuronal regulation and disorders of the brain.

In the future, p75 mRNA and protein levels will be analyzed in NRIF knockout brains using Western blot analysis and RT-PCR to determine if the increase in p75 cell number correlates to an increase in p75 protein level and transcript. Another future direction will be to count the number of cholinergic neurons in the brain and to see if new p75 positive neurons are also cholinergic. P75 knockout mice (mice lacking p75) have been reported to have more cholinergic neurons relative to wild type mice [8], suggesting that the receptor mediates normal apoptosis. An increase in p75 positive cells was expected in this region; however, no significant difference was detected. It is also possible that NRIF affects other neurotrophin receptors such as tropomyosin receptor kinase A (TrkA), B (TrkB), or C (TrkC). These receptors belong to the same neurotrophin receptor family as p75, and mediate survival and differentiation. They could also be analyzed in NRIF knockout brains to see changes in expression that could underlie differences in survival.

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