

A Proposed Mechanism for OTK18 Regulation of TNF- α in Response to Neurotoxic Insult

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Abstract

OTK18, a transcriptional suppressor with putative anti-retroviral properties, is found at the highest levels in severe human immunodeficiency virus type-1 encephalitic brains. The role of *OTK18* up-regulation in neuronal survival and corresponding responses of tumor necrosis factor-alpha (TNF- α) and neurotrophin 3 (NT3) was examined. Rat pheochromocytoma cells were transfected with either *pEGFP-OTK18* or empty vector, glutamate treated, and cell viability assessed. Quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay analyses showed significant up-regulation of TNF- α message and protein in *pEGFP-OTK18* transfected cells compared to controls. These results suggest a potential role for *OTK18* in regulating TNF- α and subsequent neuronal demise.

Introduction

Human immunodeficiency virus type one (HIV-1) infection causes a disease syndrome affecting both the immune and central nervous systems (CNS). After persistent HIV-1 infection, the virus transverse the blood brain barrier and causes neurological deficits, which progress for years into a disease complex known as HIV-1 associated dementia (HAD). Even though there is a correlation between neurological disease and HIV infection, the levels of virus do not always correlate to the clinical findings of neurologic deficit (Zheng and Gendelman, 1997). Neuronal loss is associated with viral infection of macrophages and microglia found in white matter, deep gray matter, and the cortex of the brain. There is an 18% loss of neurons in the frontal lobe of patients infected with HIV-1 and a 30-50% loss of neurons found in the frontal, parietal, and temporal cortexes of the brain (Gendelman et al., 1992).

Regulation of HIV-1 long terminal repeat (LTR) transcription is vital in viral production in infected cells. The cells that completely repress LTR activity will not express virally encoded proteins and will be latently infected. *OTK18*, a transcriptional suppressor, can diminish HIV-1 replication by affecting the HIV-1 LTR (Carlson et al. 2004a). It has anti-retroviral properties yet correlates with the most severe levels of HIV-1 (HIV-1 encephalitis) when tested in human pathological brain tissue (Carlson et al., 2004b). This suggests that *OTK18* may serve to regulate neurotoxins, such as tumor necrosis factor-alpha (TNF- α), in an attempt to protect the neurons during neurodestructive periods and in times of homeostasis. TNF- α is a proinflammatory cytokine that enhances HIV-1 expression and acts as a neurotoxin during HAD (Benveniste et al., 1998). It is up-regulated during HAD, which correlates to up-regulation of viral transcription, suggesting HAD is a TNF- α -mediated disorder (Budka, 1998). Other cytokines, such as interleukin-six (IL-6), IL-10, and interferon-gamma, can be regulated by TNF- α at the receptor level, downstream signaling pathways, or intraneuronal receptor crosstalk (Gendelman, 2002). In addition, TNF- α synergizes with glutamate, making it a more powerful neurotoxic agent by inhibiting glutamate uptake (Jiang et al., 2001; Zou and Crews, 2005). Neurotrophins, such as neurotrophin 3 (NT3), may be regulated by *OTK18* to keep them at homeostatic levels (Carlson et al., 2004a; Carlson et al., 2004b). NT3 is involved in promoting proliferation and differentiation of neuroblasts (Lukaszewicz et al., 2002) and plays a role in induction and support of axonal sprouting from injured neurons. Also, overexpression of NT3 induces axons to overcome or evade negative factors inhibiting growth (Zhou et al., 2003).

To gain insight into the possible role *OTK18* plays in regulating TNF- α and NT3 production, rat pheochromocytoma (PC12) cells were transfected with a vector containing *OTK18* and subjected to neurotoxic insult by glutamate. Glutamate was chosen because L-glutamate is a major excitatory neurotransmitter within the CNS thought to be directly involved with the neurological damage occurring in

patients suffering from stroke or neurodegenerative disorders such as HAD, motor neuron disease, Alzheimer's disease, and Parkinson's disease (Jiang *et al.*, 2001; Okumoto *et al.*, 2005). Two features that justify the use of PC12 cells are their response to glutamate and that nerve growth factor (NGF) is not needed for culture. PC12 cells respond to glutamate treatment *in vivo* through loss of viability, which suggests that this cell line represents a suitable model to investigate the mechanisms behind glutamate-induced delayed neuronal death (Froissard and Duval, 1994). NGF promotes the survival and differentiation of PC12 cells, yet these cells do not require NGF supplementation for use in experimentation (Angelastro *et al.*, 2000; Mesner *et al.*, 1995).

The aim of this project was to study regulation of TNF- α and NT3 due to *OTK18* up-regulation and neurotoxic insult. These data may assist in characterizing the effect *OTK18* has on the regulation of neuronal state. Currently, a mechanism of action for *OTK18*'s effect on neuronal survival remains elusive. Therefore, the data obtained allows us to provide a preliminary mechanism for neuronal maintenance due to *OTK18* up-regulation.

Materials and Methods

PC12 culture, transfection, and induction of neurotoxicity

PC12 cells (American Type Culture Collection [ATCC]; Manasses, VA, USA) were added to 10 ml HyQ-Dulbecco's modified Eagles media-reduced serum (DMEM-RS; Hyclone, Logan, UT) containing 15% heat-inactivated horse serum and 2.5% heat-inactivated fetal bovine serum (FBS) and placed at 37°C with 5% CO₂. For MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell viability assays, PC12 cells were plated in a poly-d-lysine coated 96-well plate (BioCoat Cell Environments, Bedford, MA) at a density of 1 x 10⁶ cells/mL. Cells for RNA and protein extraction were cultured in T-25 cell culture flasks at a density of 5 x 10⁶ cells/mL. Cells were transfected with either *pEGFP-OTK18* or *pEGFP-N3* (empty vector control) at a concentration of 1 μ g/mL, using GenePORTER 2® (Gene Therapy Systems, Inc., San Diego, CA, USA) according to manufacturer's protocol. The plates and flasks were placed at 37°C with 5% CO₂. Following 24 hours of incubation, the efficiency of transfection and gene expression was determined by viewing the cells using an inverted fluorescent microscope, manually counting the cells, and calculating the percent of cells fluorescing. To obtain optimal transfection efficiency (85% or greater), the cultures were placed back in the incubator for an additional 24 hours. Following 48 hours of transfection, neurotoxic insult was induced using 10 mM glutamate (Fisher, Fairlawn, NJ, USA) with the controls having no glutamate added. The exposed cells were incubated at 37°C with 5% CO₂ for 15 minutes. The cells were washed twice with fresh supplemented DMEM media and incubated at 37°C with 5% CO₂ for 24 hours.

MTT assays

Following 24 hours of neurotoxic insult, a MTT assay was performed on the 96-well plate. The plate was incubated at 37°C with 5% CO₂ for 30-45 minutes, the MTT solution was aspirated from each well, and dimethylsulfoxide (Fisher) was added to each well. The plate was incubated at room temperature for 15 minutes, and read using an Emax precision plate reader (Molecular Devices, Sunnyvale, CA, USA) at 490nm. For each gene tested, a one-tailed student's *t*-test for unpaired data with an alpha level of 0.05 was performed using Microsoft Office Excel version 2003.

RNA extraction and qRT-PCR analyses

RNA was extracted from each of the T-25 flasks and purified utilizing the Qiagen RNeasy Plus Mini® kit according to manufacture's instructions (Qiagen Inc., Valencia, CA). qRT-PCR was performed using Taqman Gene Expression Assay® kits and the 7500 Real Time PCR® system (Applied Biosystems, Foster City, CA) according to manufacture's instructions. The primer and probe sets used were *TNF- α* (assay #Rn99999017_m1), *NT3* (assay #Rn01199850_m1), *OTK18* (assay #Hs00232535_m1), and *GAPDH* (endogenous control; assay #Rn99999916_s1). Reactions were carried out in triplicate and performed in a 50 μ l volume utilizing 200 ng total RNA sample and TaqMan® One-Step RT-PCR Mix (Applied Biosystems). Negative controls without RNA for each primer/probe set were also run. Cycling

parameters included 48°C for 30 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The PCR products were analyzed in the linear range for amplification with *GAPDH* using the 7500 Real Time PCR System Sequence Detection Software® (Applied Biosystems). The relative quantitative results were used to determine changes in gene expression in *pEGFP-OTK18* and *pEGFP-N3* transfected PC12 cells supplemented with glutamate compared to those not supplemented on a log₂ scale per manufacturer's instructions (Applied Biosystems). For each gene tested, a one-tailed student's *t*-test for unpaired data with an alpha level of 0.05 was performed using Microsoft Office Excel version 2003.

TNF- α ELISA

An ELISA was performed for quantitative determination of TNF- α protein concentrations in PC12 cell culture supernatants according to manufacturer's instructions (R & D systems; Minneapolis, MN). The culture supernatants were diluted to 40 ng/ μ L and run in triplicate. Plates were read at an absorbance of 450 nm with wavelength correction at 540 nm utilizing a Multiskan Ex® plate reader (Thermo Electron Corporation, Vantaa, Finland). Comparative analysis was performed using Ascent® software (Thermo Electron Corporation). One-tailed student's *t*-test for unpaired data with an alpha level of 0.05 was performed using Microsoft Office Excel version 2003.

RESULTS

PC12 cell viability was significantly decreased ($p < 0.05$) with the addition of glutamate. Cell viability was significantly different in cells transfected with either *pEGFP-OTK18* or the control plasmid (*pEGFP-N3*) exposed to glutamate as compared to the minus glutamate controls (Fig. 1). qRT-PCR of *OTK18* mRNA levels in PC12 cells was significantly up-regulated ($p < 0.05$) in PC12 cells transfected with *pEGFP-OTK18* compared to those transfected with the control plasmid (*pEGFP-N3*) whether or not glutamate was added (data not shown). qRT-PCR data is shown as a ratio of *pEGFP-OTK18* to *pEGFP-N3* transfected cells, which represents the average fold increase on a log₂ scale for *NT3* or *TNF- α* expression in PC12 cells supplemented with glutamate compared to those not supplemented with glutamate. A significant

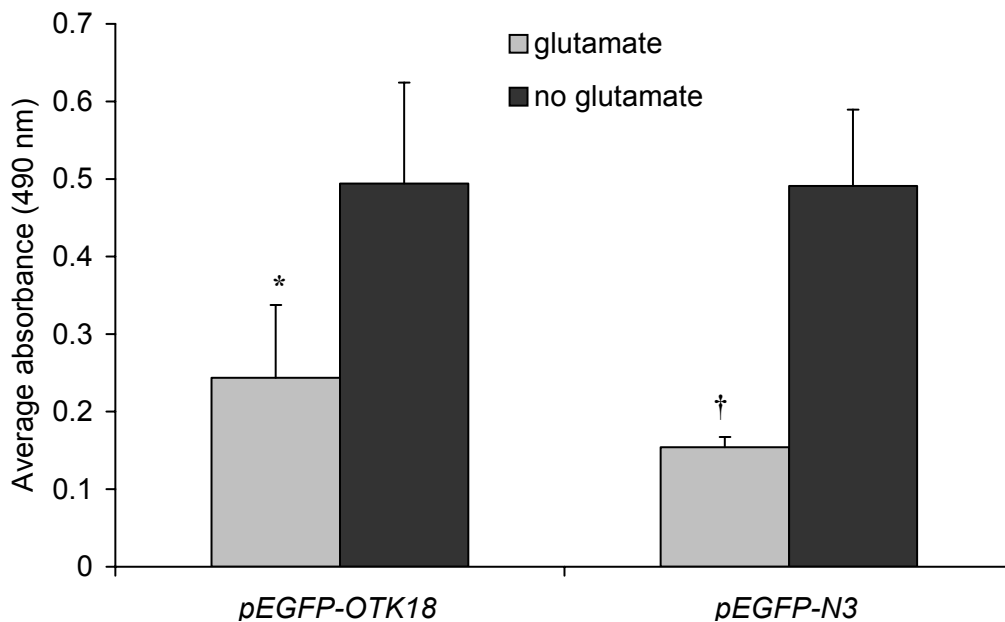


Figure 1. MTT assay of PC12 cells transfected with *pEGFP-OTK18* or *pEGFP-N3* \pm glutamate. * represents comparison between the plus and minus glutamate measurements for the *pEGFP-OTK18* condition ($p < 0.05$), and † represents comparison between the \pm glutamate measurements for the *pEGFP-N3* condition ($p < 0.05$). Error bars represent standard error of the mean (SEM) and $n = 8$.

increase ($p < 0.05$) in $TNF-\alpha$ mRNA levels was seen in glutamate treated cells versus untreated controls, whereas $NT3$ mRNA levels were not significantly changed (Fig. 2A). The effect of glutamate on the level of $NT3$ and $TNF-\alpha$ expression is presented as a ratio of the plus glutamate to the minus glutamate conditions for the $pEGFP-OTK18$ and $pEGFP-N3$ transfected PC12 cells on a \log_2 scale. $TNF-\alpha$ was significantly up-regulated ($p < 0.05$) in $pEGFP-OTK18$ transfected cells versus those transfected with empty vector ($pEGFP-N3$), whereas $NT3$ levels were not significantly changed (Fig. 2B).

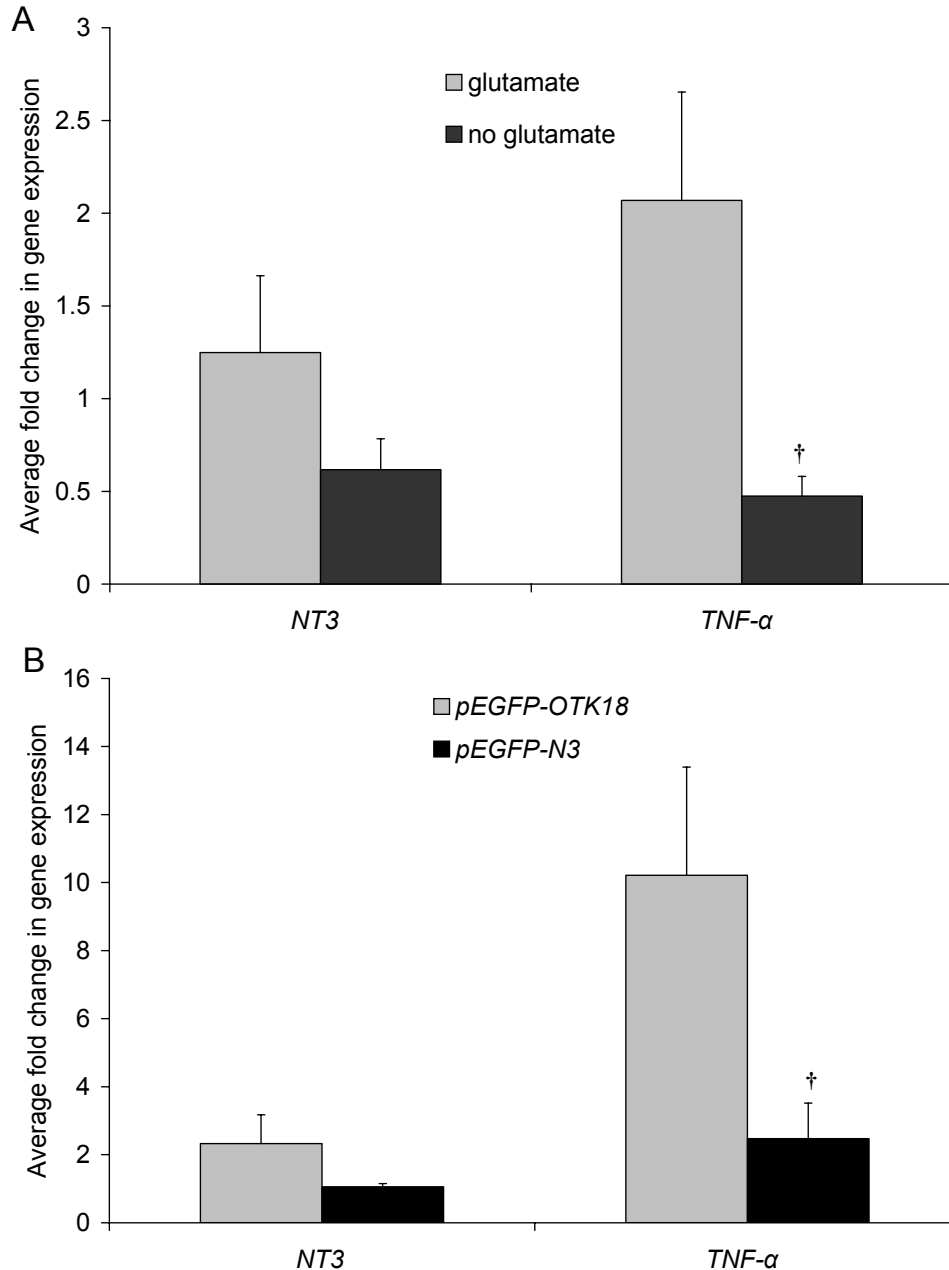


Figure 2. Quantitative gene expression of $NT3$ and $TNF-\alpha$ assessed by qRT-PCR measured as average fold change on a \log_2 scale. A). To examine the effect of glutamate on $TNF-\alpha$ or $NT3$ expression, results are a ratio of $pEGFP-OTK18$ to $pEGFP-N3$ transfected PC12 cells \pm glutamate. † is the comparison between \pm glutamate measurements for the ratio of $pEGFP-OTK18$ to $pEGFP-N3$ transfected PC12 cells for $TNF-\alpha$ ($p < 0.05$). B). To examine the effect of OTK18 or N3 on $TNF-\alpha$ or $NT3$ expression, the results are a ratio of the plus glutamate to the minus glutamate of the $pEGFP-OTK18$ to $pEGFP-N3$ transfected PC12 cells. † is the comparison of the $pEGFP-OTK18$ to $pEGFP-N3$ for $TNF-\alpha$ ($p < 0.05$). Error bars represent SEM and $n = 3$.

ELISA (Fig. 3) demonstrated a significant increase ($p < 0.05$) of TNF- α levels in PC12 cells transfected with *pEGFP-OTK18* and supplemented with glutamate compared to unsupplemented controls. There was significant increase of TNF- α levels in the *pEGFP-OTK18* plus glutamate when compared to *pEGFP-N3* plus glutamate ($p < 0.05$). Significant increased TNF- α levels was also seen in the *pEGFP-OTK18* minus glutamate culture compared to the *pEGFP-N3* plus glutamate ($p < 0.05$).

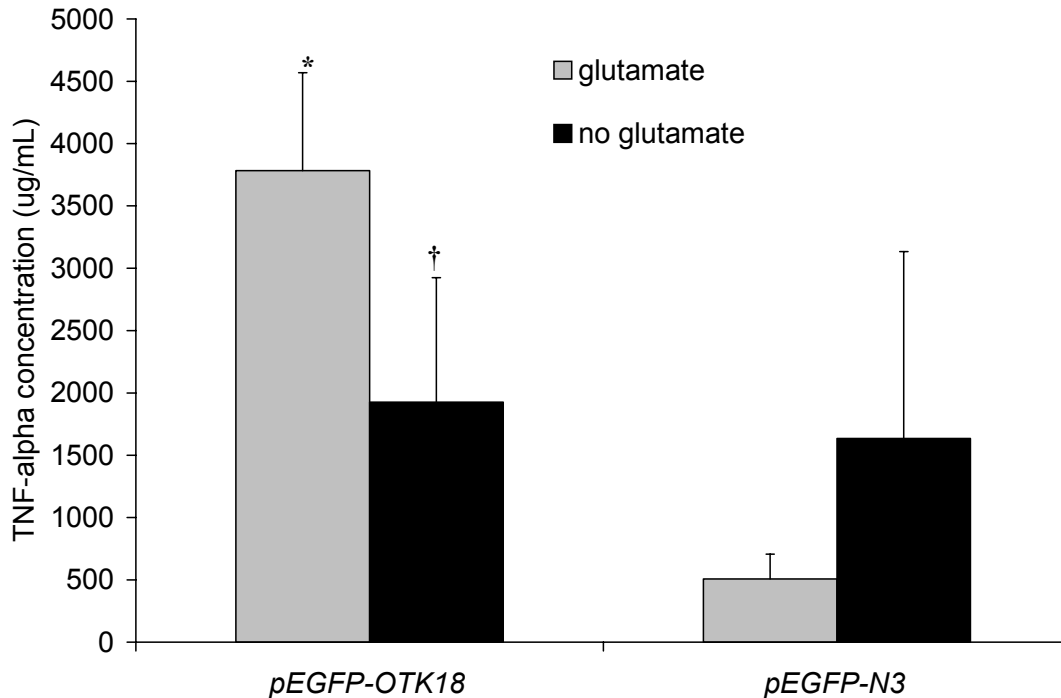


Figure 3. TNF- α ELISA detection in *pEGFP-OTK18* and *pEGFP-N3* transfected PC12 cell culture supernatants \pm glutamate. The * represents comparison to *pEGFP-OTK18* plus glutamate to all other conditions ($p < 0.05$) and † represents comparison to *pEGFP-OTK18* minus glutamate to *pEGFP-N3* plus glutamate ($p < 0.05$). Error bars represent SEM and $n = 3$.

Discussion

This study provides insight into the possible functionality of *OTK18*. *OTK18* is a novel gene and not much is known about its function during HIV-1 infection, inflammation, or homeostasis. During normal states, *OTK18* is expressed in all body tissues (Carlson *et al.* 2004a). In post-mortem brain samples with severe HIVE, *OTK18* is up-regulated and localized to the mononuclear phagocytes (Carlson *et al.* 2004b). The mechanism of action for *OTK18* remains elusive, but this study has allowed a preliminary insight into a possible functionality of the gene.

In this study, a significant decrease in cell viability was found in both the *pEGFP-OTK18* and *pEGFP-N3* transfected cell cultures exposed to 10 mM glutamate. This may be explained by the accumulation of oxidants, because the ability to scavenge the free radicals is impaired in PC12 cells following exposure to glutamate (Pereira and Oliveira, 1997). TNF- α ELISA showed significant decreased levels of TNF- α in all conditions compared to *pEGFP-OTK18* plus glutamate. There was also significant decreased levels of TNF- α in the *pEGFP-N3* plus glutamate compared to the *pEGFP-OTK18* minus glutamate for PC12 cell culture. Taken together, the qRT-PCR and ELISA results indicate a significant up-regulation of both TNF- α message and TNF- α protein in *pEGFP-OTK18* transfected PC12 cells exposed to neurotoxic insult compared to control.

The results from this study may suggest that glutamate neurotoxicity leads to *OTK18* induced up-regulation of TNF- α expression, leading to increased levels of TNF- α protein. Once a threshold of glutamate is reached, *OTK18* may work through negative feedback by suppressing the expression of

TNF- α and up-regulating the expression of neurotrophins, such as *NT3*. The expression of neurotrophins could counteract the effects of both *TNF- α* and glutamate leading to increased neuronal survival (Fig. 4). Since this study was not performed using HIV-1 infected cells, the data may also suggest that what is occurring may be a generalized feedback inhibition in regards to inflammation and not specifically HIV-1 infection. This mechanism is preliminary, but does provide a starting point for future investigation.

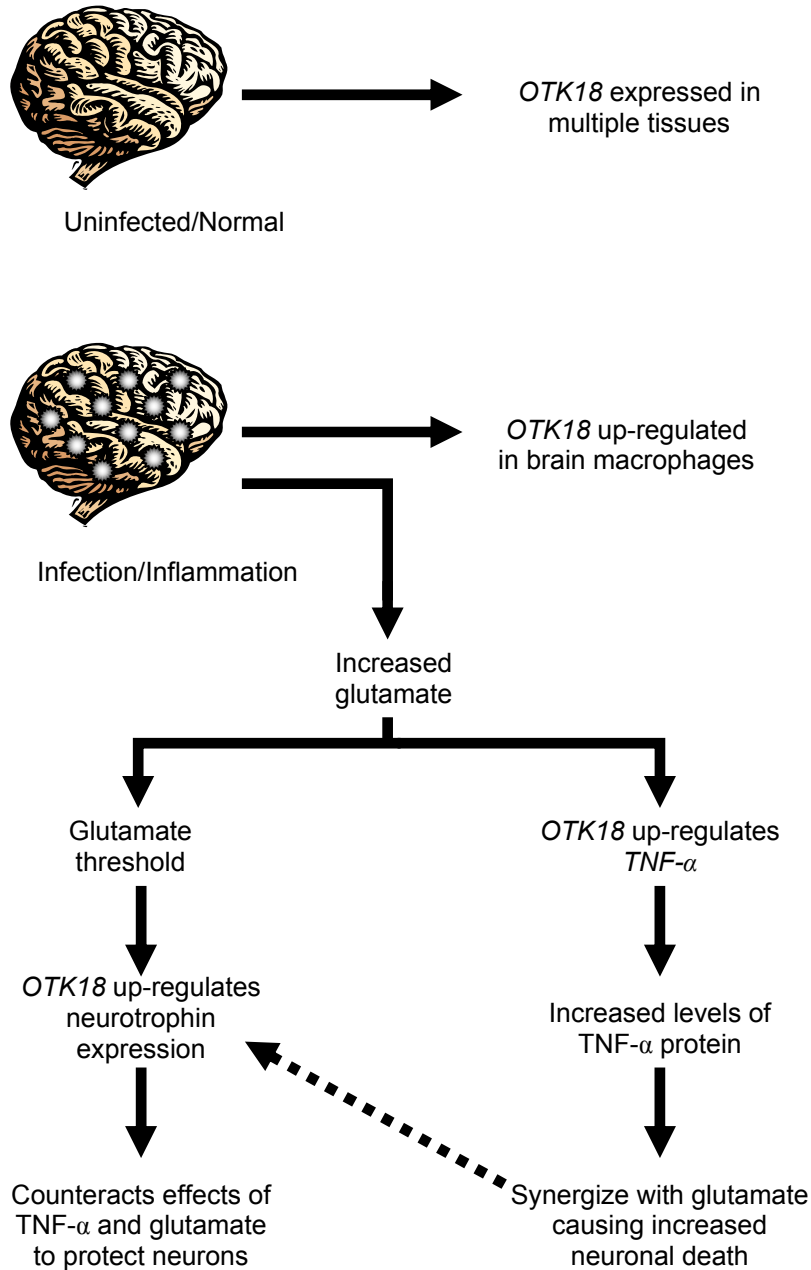


Figure 4. Flow chart representing a proposed mechanism for OTK18 during uninfected/normal versus infected/inflamed states. The dashed arrow represents a proposed negative feedback mechanism for OTK18 regulation of *TNF- α* .

Showing that *pEGFP-OTK18* transfected PC12 cell culture produces increased levels of TNF- α protein and message levels during neurotoxic insult adds to what little is known about this gene. Further experimentation is necessary to determine the quantity of NT3 protein produced by the cultures. Also, other neurotoxins and neurotrophins should be investigated for up-regulation, including NGF, which is a neurotrophin that promotes survival, differentiation, and growth of neuronal cells (Bai and Kusiak, 1997; Nomoto *et al.*, 2000). When neurons are exposed to glutamate, depolarization occurs causing the regulated release of more neurotrophins, including NGF, NT3, and brain-derived neurotrophic factor (BDNF) (Blochl and Thoenen, 1996). It is thought that cells transfected with a neurotrophin, like NGF, can induce the regulated release of other neurotrophins, including BDNF and NT3, resulting in a positive feedback loop. This feedback loop results in the amplification of the neurotrophin in focusing the release of other neurotrophins at subcellular locations where receptors for these proteins are present (Kruttschgen *et al.*, 1998). This mechanism could be studied further by repeating the aforementioned experiments and supplementing the PC12 culture media with NGF.

Further research involving *OTK18* could provide insight into the neuropathology of HIV-1 infection or into inflammation in general. If *OTK18* does indeed work via a negative feedback mechanism, this gene could be a possible target for drug therapy. If *OTK18* does not work through negative feedback, determining its exact mechanism of action during disease progression or inflammation would assist development of treatments for HIV-1 infection or generalized inflammation.

Acknowledgements

Dr. Paul Twigg, Dr. Janet Steele, and Teresa Donze for technical support and intellectual input. Three anonymous reviewers with helpful comments and editing. This research was supported by grant number P20 RR016469 from the INBRE Program of the National Center for Research Resources a component of the National Institutes of Health, the University of Nebraska at Kearney (UNK) Research Services Council (RSC) Creative Activity Grant, UNK-RSC Graduate Research Grant, UNK-RSC Undergraduate Research Grant, UNK College of Natural and Social Sciences, and the UNK Biology Department.

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