Proceedings of the 4th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/76/2024.19966

Experimental Design: Investigating the Role of Autophagy and BDNF in Neuronal Death Under Chronic Stress

Evelyn Yilin Chen[1,4,*], Yiru Chen[2,5], Yuqian Lin[3,6]

[1]Department of Biochemistry, University of Toronto, Toronto, M5S 1A1, Canada
[2]Department of Clinical Medicine, Shandong First Medical University, Jinan, 250117, China
[3]Shunyi Beanstalk bilingual school, Shunyi Beanstalk bilingual school, Beijing, 101318, China

[4]evelyn.yilin.chen@outlook.com
[5]a260968401@163.com
[6]nissalin2018@163.com
*corresponding author

Abstract. Chronic stress impairs adult hippocampal neurogenesis (AHN) by reducing the survival and proliferation of neural stem cells (NSCs) in the dentate gyrus. Central to this process is cortisol, which interacts with the glucocorticoid receptor (GR) and represses Brain-Derived Neurotrophic Factor (BDNF) transcription, leading to NSC death and disrupted AHN. Autophagy, a cellular degradation process, plays a dual role in cell survival and death under chronic stress. This study investigates the interplay between cortisol, BDNF, and autophagy in influencing NSC viability and AHN. Using human iPSC-derived NSCs, we explore whether GR activation reduces BDNF levels, leading to increased autophagy and cell death, or if other pathways are involved. The study also determines whether cortisol impacts BDNF synthesis, its signaling via the TrkB receptor, or both. Our research employs genetic manipulations, cortisol treatments, and assays to quantify autophagy and cell viability under stress. Understanding these mechanisms could provide insights into stress-related neuropsychiatric disorders and inform therapeutic strategies to enhance brain resilience and function under chronic stress.

Keywords: Chronic stress, Cortisol, glucocorticoid receptor (GR), Brain-Derived Neurotrophic Factor (BDNF), Autophagy, adult hippocampal neurogenesis (AHN).

1. Introduction

1.1. Background Information

Chronic stress impairs adult hippocampal neurogenesis (AHN) by reducing the survival and proliferation of neural stem cells (NSCs) in the dentate gyrus. A key player in this process is cortisol, a glucocorticoid that interacts with the glucocorticoid receptor (GR). GR binding near exon IV of the Brain-Derived Neurotrophic Factor (BDNF) gene represses BDNF transcription [1]. This reduction in BDNF levels leads to NSC death and impaired AHN, which are essential for cognitive functions and emotional regulation [2,3].

DOI: 10.54254/2753-8818/76/2024.19966

While about 50% of NSCs and early neural progenitor cells naturally undergo apoptosis, chronic stress can induce NSC decline through autophagy [4]. Autophagy, a process regulated by autophagy-related (ATG) proteins and the mechanistic target of rapamycin (mTOR), involves degrading cytoplasmic components and damaged organelles via lysosomes [5]. Typically protective, autophagy can also contribute to cell death. Studies have shown that deleting a key autophagic gene in mice partially restores AHN under stress conditions, though the exact link between GR activation, autophagy, and NSC viability remains unclear.

BDNF promotes neurogenesis by interacting with the TrkB receptor, activating pathways crucial for neuronal growth, differentiation, and synaptic plasticity [6-8]. BDNF also enhances neuronal resistance to mitochondrial dysfunction, induces anti-oxidative enzymes, and increases the expression of anti-apoptotic proteins like Bcl-2 [7]. Additionally, BDNF regulates autophagy to maintain cellular homeostasis, promoting selective degradation of damaged components and inhibiting excessive autophagy via the mTOR pathway [8].

1.2. Cell Line Justification

To investigate the effects of chronic stress on AHN, we employed human iPSC-derived NSCs as our model. These cells are particularly pertinent due to their unique response to stress—undergoing autophagy rather than apoptosis, unlike other neurons. This model offers the dual advantage of mimicking natural NSC behaviours while facilitating detailed genetic and biochemical analyses [9]. Notably, these NSCs possess functional glucocorticoid receptors, which are critical for cortisol's effects, and express TrkB receptors, enabling the study of BDNF-mediated pathways [7,10]. This approach ensures a focused examination of stress-related mechanisms in a controlled, reproducible environment.

1.3. Motivation & Objectives

The relationship between chronic stress, cortisol, BDNF, and NSC loss is complex. It is hypothesized that cortisol impairs BDNF production or signalling, leading to reduced BDNF levels and disrupted autophagy function, resulting in autophagy-induced cell death [4-9]. Competing evidence suggests BDNF deficiency might directly cause neuronal death, with autophagy further reducing BDNF levels, creating a detrimental feedback loop [10].

This study aims to investigate how cortisol, BDNF, and autophagy interact to influence NSC viability and AHN. The primary research questions are:

1. Define the interplay between GR activation, BDNF levels, and autophagy in NSC death.
2. Determine whether cortisol impacts BDNF synthesis, its signalling via the TrkB receptor, or both, in neural cells.

This research addresses critical gaps in understanding how chronic stress affects brain function. By elucidating these mechanisms, we can develop targeted treatments to enhance NSC survival and function, improving outcomes for individuals experiencing chronic stress and its neuropsychiatric consequences.

2. Experimental Design Overview

2.1. Objective 1: To determine whether BDNF downregulation through GR activation directly leads to increased autophagy and cell death, or if other pathways are also involved.

This part of the study seeks to determine if the downregulation of BDNF by GR activation directly leads to increased autophagy and cell death or if GR impacts these processes through other pathways. Understanding these mechanisms will enhance our insight into potential therapeutic targets for stress-related neurological disorders.

To investigate these relationships, we developed a rigorous experimental framework. Human iPSC-derived NSCs are cultured under controlled conditions with genetic manipulations to modulate GR and BDNF activity—knocking down BDNF via RNA interference (RNAi) or overexpressing it using a high copy number promoter, combined with cortisol treatments to activate GR, mimicking chronic stress.

DOI: 10.54254/2753-8818/76/2024.19966

We utilize multiple assays to quantify the effects of chronic stress on autophagy and cell viability in NSCs. Autophagy activity is assessed via immunofluorescence microscopy to detect LC3-II and p62, markers of autophagosome formation and selective autophagy, respectively [10]. An increase in LC3 puncta per cell indicates enhanced autophagy. NSC viability is measured using the Trypan Blue exclusion assay, providing quantitative data by distinguishing live from dead cells. To confirm NSC death is not due to apoptosis, we employ a Caspase-3 activity assay. Additionally, RNA sequencing (RNA-seq) is used to analyse gene expression changes, particularly how GR activation and BDNF levels influence autophagy-related genes and BDNF itself, elucidating regulatory pathways contributing to cell death.

Our experimental design explores GR and BDNF effects on NSC viability and autophagy across five conditions:

1. Baseline Control: No treatment to establish a reference for normal NSC function.
2. CORT Natural Effect: Cells treated solely with cortisol to evaluate GR activation effects.
3. BDNF Knockdown: Isolates the effect of reduced BDNF on autophagy, independent of cortisol.
4. CORT with BDNF Overexpression: Examines the interaction between elevated BDNF levels and cortisol treatment.
5. Exaggerated Effect: Combines cortisol treatment and BDNF knockdown to assess synergistic effects on NSC function and autophagy.

Our expected results aim to clarify the roles of BDNF and GR activation in regulating autophagy and cell death under stress. If BDNF knockdown mimics cortisol treatment, it indicates reduced BDNF alone triggers autophagy and cell death. If BDNF overexpression with cortisol prevents these effects, it suggests GR does not affect autophagy through BDNF-independent pathways, confirming BDNF's direct regulation.

If combined treatments of increased cortisol and reduced BDNF lead to more severe outcomes than single treatments, it indicates a synergistic effect. Comparing cells with only GR activation to those with both GR activation and BDNF knockdown will validate this synergy. Enhanced autophagy in the combined treatment would suggest a joint effect of GR and BDNF on autophagy pathways. This study aims to define how GR and BDNF modulate autophagy under stress.

2.2. Objective 2: To determine whether cortisol impacts BDNF synthesis, its signalling via the TrkB receptor, or both, in neural cells.

The objective of this study is to determine whether cortisol impacts BDNF synthesis, its signalling via the TrkB receptor, or both, in neural cells. While evidence shows cortisol reduces BDNF levels, it is unclear if cortisol directly affects TrkB receptor activities. Understanding this relationship is crucial for comprehending the stress response at a molecular level and could offer insights into brain resilience mechanisms against stress. Neurons are the primary producers and recipients of BDNF, making this study relevant for AHN using neuronal stem cells.

To address this objective, we will culture iPSC-derived NSCs under standardized conditions and expose them to cortisol to simulate chronic stress scenarios. The effects of cortisol will be quantitatively assessed through several measures, including cell viability, autophagy induction, and activation of the TrkB signaling pathway. Western blotting will be employed to evaluate the phosphorylation levels of key proteins involved in these pathways, specifically phosphorylated ERK1/2 (p-ERK1/2), Akt (p-Akt) at Ser473, and PLCγ (p-PLCγ). These proteins were selected based on their established roles in TrkB signalling p-ERK1/2 is crucial for neuronal survival and synaptic plasticity, p-Akt represents the activation of the PI3K pathway which supports neuronal survival and growth, and p-PLCγ, which facilitates the mobilization of intracellular calcium and activation of PKC, is indicative of TrkB receptor engagement [9, 11].

The experimental design includes four treatment conditions:

1. Basal Level (No Cortisol): Control to establish baseline levels of BDNF synthesis, TrkB signaling, and cellular health.

DOI: 10.54254/2753-8818/76/2024.19966

2. Cortisol Only: Cells treated with cortisol to determine its effects on BDNF synthesis and TrkB signaling.
3. Cortisol + Exogenously Provided BDNF: Cells treated with cortisol and supplemented with exogenous BDNF to see if BDNF addition counteracts cortisol effects.
4. TrkB Inhibitor/Antagonist: Cells treated with a TrkB inhibitor/antagonist for varying durations to evaluate the impact of cortisol on TrkB signalling.

Expected outcomes will help delineate interactions between BDNF, autophagy, and TrkB signalling under cortisol. If exogenous BDNF reduces autophagy, increases cell count, and normalizes TrkB activation, it suggests symptoms are due to reduced BDNF. If not, it indicates cortisol also affects TrkB signalling. Western blot analysis will further elucidate the specific pathways affected by cortisol. This design aims to define the roles of cortisol, BDNF synthesis, and TrkB receptor activities in modulating autophagy and cell viability under stress.

3. Methods (Objective 1)

Preparation and Culture of iPSC-Derived Neural Stem Cells. Human iPSC-derived neural stem cells (NSCs) from the Healthy Control Human iPSC Line, Female, SCTi003-A, were cultured using the STEMdiff™ SMADi Neural Induction Kit (Catalog #08581). Cultureware was pre-coated with poly-L-ornithine and laminin. Cells were maintained in STEMdiff™ Neural Progenitor Medium (Catalog #05833) to promote CNS markers such as PAX6, SOX1, and Nestin, while minimizing SOX10. Cultures were kept at 37°C in a humidified 5% CO2 atmosphere. Passaging was performed at 80% confluence with daily monitoring to ensure cells remained undifferentiated [7, 12] .

Cortisol Treatment. Cortisol (Sigma Aldrich) was dissolved in ethanol to create a 100 mM stock solution, stored at -20°C. For long-term exposure, cells received 5 μM cortisol (0.0005% ethanol) every 24 hours for seven days, with regular cell count checks [13].

BDNF Knockdown and Overexpression in NSC Culture. To knock down BDNF, we used MISSION® shRNA Plasmid DNA (Sigma-Aldrich, Catalog No. TRCN0000116073). Neurons were maintained at 37°C in a 5% CO2 atmosphere with neuron-specific media. Transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific) per the manufacturer's protocol. BDNF knockdown efficiency was assessed via quantitative PCR and Western blot. Cell health was monitored daily, with medium changes every 2-3 days. Functional assays evaluated the impacts of BDNF reduction on cell viability, neurite outgrowth, and synaptic functionality [11, 12].

For BDNF overexpression, a lentiviral vector with the BDNF coding DNA sequence under a CMV promoter was constructed. Lentiviral particles were produced in HEK293T cells transfected with the BDNF construct and necessary packaging plasmids using Lipofectamine 2000. Viral supernatants were harvested 48-72 hours post-transfection and filtered through a 0.45 μm filter. Primary neurons were plated a day before adding the lentiviral supernatant with 8 μg/ml polybrene. After 48 hours, the medium was supplemented with a selective antibiotic to isolate cells expressing BDNF. Overexpression was confirmed by Western blot [7, 11, 12].

RNA-seq. Cells were lysed using 350 μl of Qiagen Buffer RLT Plus (Qiagen, 1053393). Total RNA was isolated with the Qiagen RNeasy Plus Mini Kit (74134). RNA integrity and concentration were assessed using the Invitrogen Qubit RNA BR Assay Kit (Q10211) and the Agilent RNA ScreenTape system (5067-5576). For sequencing, 200 ng of RNA was processed using the NEBNext Ultra II RNA Library Prep Kit for Illumina (E7770). Libraries were barcoded with NEBNext Multiplex Oligos (E7335, E7500), quantified using the KAPA Library Quantification Kit (KK4824), and evaluated with the Agilent 4200 TapeStation system. Sequencing was conducted on an Illumina NovaSeq 6000, producing single-end 100 bp reads for comprehensive transcript quantification [14].

Cell Viability Assessment using Trypan Blue Assays. Cells were resuspended in PBS, mixed with 0.4% Trypan Blue, and incubated at room temperature for 2-3 minutes. Using a hemocytometer under light microscopy at 100x magnification, viable cells, which exclude the dye and remain clear, were distinguished from non-viable, dye-absorbing blue cells [7, 15].

DOI: 10.54254/2753-8818/76/2024.19966

Immunofluorescence for Measuring Autophagy. iPSC-derived human brain stem cells were fixed with 4% PFA in PBS for 15 minutes at room temperature after reaching 70-80% confluence, washed with PBS, permeabilized with 0.2% Triton X-100 for 10 minutes, and blocked with 5% normal donkey serum for 1 hour. Cells were incubated overnight at 4°C with rabbit anti-LC3-II (1:1000), mouse anti-p62 (1:500), and rabbit anti-cleaved caspase-3 (1:400) antibodies. After washing, cells were treated with donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 555 secondary antibodies for 1 hour in the dark. Finally, cells were stained with DAPI and mounted using VECTASHIELD. Imaging was performed using a Nikon A1R Confocal Microscope with FCS + FLIM capabilities for precise localization and quantification of autophagy markers [4,5,8,9,10].

4. Methods (Objective 2)

For *Cell Culture*, *Cortisol Treatment*, *Cell Count Measurement*, and *Autophagy Activity Assessment*, please refer to method section for Objective 1.

Treatment with TrkB Antagonist/Inhibitor. To investigate the effects of TrkB inhibition, iPSC-derived NSCs were treated with the TrkB antagonist ANA-12 (10 μM) and the TrkB inhibitor K252a (100 nM), both dissolved in DMSO. Control groups received an equivalent volume of DMSO. Treatments were administered for 24 hours [16] .

Exogenous BDNF Supplement. Recombinant human BDNF protein (Thermo Fisher Scientific, Catalog No. PHC7074) was reconstituted in sterile PBS, aliquoted, and stored at -80°C. An aliquot was thawed and diluted in pre-warmed culture medium to 50 ng/ml. The existing medium was replaced with the BDNF-supplemented medium, and cells were incubated at 37°C in a 5% CO_2 humidified incubator [12] .

Protein Extraction and Quantification. After treatment, cells were washed with cold PBS and lysed with RIPA buffer supplemented with protease and phosphatase inhibitors. Lysates were incubated on ice for 30 minutes and centrifuged at 14,000 g for 15 minutes at 4°C. Protein concentration was measured using the BCA protein assay kit [17].

SDS-PAGE and Western Blotting. Equal amounts of protein (20-40 μg) were mixed with 4× Laemmli sample buffer, boiled for 5 minutes, and loaded onto a 10% SDS-PAGE gel. Electrophoresis was performed at 120V until the dye front reached the bottom of the gel. Proteins were transferred to a PVDF membrane using a wet transfer system at 100V for 1 hour. The membrane was blocked in 5% BSA in TBST for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies: anti-phospho-ERK1/2 (1:1000), anti-ERK1/2 (1:1000), anti-phospho-Akt (1:1000), anti-Akt (1:1000), anti-phospho-PLCγ (1:1000), and anti-PLCγ (1:1000). The membrane was washed with TBST and incubated with HRP-conjugated secondary antibodies (1:5000) in 5% BSA in TBST for 1 hour at room temperature. After washing, the chemiluminescent signal was developed using an ECL detection reagent and captured with a chemiluminescent imaging system [7, 12] .

5. Evaluation & Conclusion

While iPSC-derived NSCs are powerful tools for studying cellular mechanisms, they cannot fully replicate the complexity of in vivo brain environments, such as the blood-brain barrier, systemic hormonal levels, and cell-cell interactions. Results from specific iPSC-derived NSC lines may not translate across all NSC types due to genetic variability and individual stress responses. Additionally, focusing on autophagy and BDNF in the context of cortisol might overlook other critical pathways like inflammation.

Future studies should integrate these findings into more complex stress models, using animal models or organoids that better mimic human brain environments. Translational studies should validate potential drug targets and autophagy modulators in clinical settings to treat or prevent stress-related neurodegenerative conditions. Further research could explore interactions between autophagy and other stress-response mechanisms like oxidative stress, inflammation, and mitochondrial dysfunction, potentially uncovering multi-target therapeutic strategies.

DOI: 10.54254/2753-8818/76/2024.19966

In conclusion, this study elucidates the complex interactions between chronic stress, cortisol, BDNF, and autophagy, focusing on their effects on NSC viability and AHN. By investigating autophagy-induced cell death and cortisol's impact on BDNF synthesis and TrkB receptor signalling, we aim to address critical gaps in understanding how stress impacts brain function. The findings could enhance therapeutic strategies for depression and significantly contribute to knowledge of neurodegenerative diseases.

References

[1] Chen, H., Lombès, M., & Le Menuet, D. (2017). Glucocorticoid receptor represses brain-derived neurotrophic factor expression in neuron-like cells. *Molecular Brain, 10*(1), 12.

[2] Snyder, J. S., Soumier, A., Brewer, M., Pickel, J., & Cameron, H. A. (2011). Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature, 476*(7361), 458-461.

[3] Lucassen, P. J., Oomen, C. A., van Dam, A. M., & Meerlo, P. (2014). Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. *European Neuropsychopharmacology, 24*(5), 721-734.

[4] Jung, S., Choe, S., Woo, H., Jeong, H., An, H. K., Moon, H., Ryu, H. Y., Yeo, B. K., Lee, Y. W. , Choi, H., Mun, J. Y., Sun, W., Choe, H. K., Kim, E. K., & Yu, S. W. (2020). Autophagic death of neural stem cells mediates chronic stress-induced decline of adult hippocampal neurogenesis and cognitive deficits. *Autophagy, 16*(3), 512-530.

[5] Mizushima, N. (2007). Autophagy: Process and function. *Genes & Development, 21*(22), 2861-2873.

[6] Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: Roles in neuronal development and function. *Annual Review of Neuroscience, 24*, 677-736.

[7] Numakawa, T., Adachi, N., Richards, M., Chiba, S., & Kunugi, H. (2010). BDNF function and intracellular signaling in neurons. *Histology and Histopathology, 25*(2), 237-258.

[8] Smith ED, Prieto GA, Tong L (2014) Rapamycin and interleukin-1β impair brain-derived neurotrophic factor-dependent neuron survival by modulating autophagy. J Biol Chem. 289(30):20615-20629.

[9] Nagayach, A., & Wang, C. (2024). Autophagy in neural stem cells and glia for brain health and diseases. *Neural Regeneration Research, 19*(4), 729-736.

[10] Zhang, K., Wang, F., Zhai, M., He, M., Hu, Y., Feng, L., Li, Y., Yang, J., & Wu, C. (2023). Hyperactive neuronal autophagy depletes BDNF and impairs adult hippocampal neurogenesis in a corticosterone-induced mouse model of depression. Theranostics, 13(3), 1059–1075.

[11] Quan, J.-H., Ismail, H. A. H. A., Cha, G.-H., Jo, Y.-J., Gao, F. F., Choi, I.-W., Chu, J.-Q., Yuk, J.-M., & Lee, Y.-H. (2020). VEGF production is regulated by the AKT/ERK1/2 signaling pathway and controls the proliferation of *Toxoplasma gondii* in ARPE-19 cells. *Frontiers in Cellular and Infection Microbiology, 10*, 184.

[12] Almeida, R. D., Manadas, B. J., Melo, C. V., Gomes, J. R., Mendes, C. S., Grãos, M. M., Carvalho, R. F., Carvalho, A. P., & Duarte, C. B. (2005). Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death and Differentiation, 12*(10), 1329–1343.

[13] Heard, K. J., Shokhirev, M. N., & Becronis, C. (2021). Chronic cortisol differentially impacts stem cell-derived astrocytes from major depressive disorder patients. *Translational Psychiatry, 11*, 608.

[14] Jiang, P., Hou, Z., Bolin, J. M., Thomson, J. A., & Stewart, R. (2017). RNA-Seq of human neural progenitor cells exposed to lead (Pb) reveals transcriptome dynamics, splicing alterations and disease risk associations. *Toxicological Sciences, 159*(1), 251-265.

[15] Mani, S., & Swargiary, G. (2023). In vitro cytotoxicity analysis: MTT/XTT, trypan blue exclusion. In *Animal Cell Culture: Principles and Practice. Techniques in Life Science and Biomedicine for the Non-Expert*. Springer, Cham.

DOI: 10.54254/2753-8818/76/2024.19966

[16] Cazorla, M., Premont, J., Mann, A., Girard, N., Kellendonk, C., & Rognan, D. (2011). Identification of a low-molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice. *The Journal of Clinical Investigation, 121*(5), 1846-1857.

[17] Reinmuth-Selzle K, Tchipilov T, Backes AT, et al. (2022) Determination of the protein content of complex samples by aromatic amino acid analysis, liquid chromatography-UV absorbance, and colorimetry. Anal Bioanal Chem.414(15):4457-4470.