

Orexin-Induced Upregulation of *OX1R* And *BDNF* Suggests an Adaptive Neuroprotective Mechanism Against Oxidative Stress

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ABSTRACT: Chronic sleep disturbance elevates reactive oxygen species (ROS) and lowers brain-derived neurotrophic factor (BDNF), predisposing the brain to injury. Since orexin (hypocretin) maintains wakefulness and activates stress-responsive pathways, we investigated whether orexin signaling imparts glial resilience to oxidative stress. This study investigated whether lentiviral HCRT overexpression protects A172 glial cells against hydrogen peroxide (H₂O₂)-induced oxidative stress by altering orexin receptor signaling, apoptosis-related responses, and BDNF-associated neuroprotective gene expression. Lentivirus encoding prepro-orexin (*HCRT*) was used to transduce human A172 glial cells. Control groups included empty-virus and no-virus groups. The overexpression of *HCRT* was dose-dependent and was also associated with a significant increase in *OX1R* and *BDNF*. Functionally, orexin enhanced cell viability and diminished apoptosis in response to oxidative stress in a dose-dependent manner, signifying cytoprotection. These synchronized molecular and functional alterations indicate the activation of *OX1R*-dependent pro-survival pathways (cAMP/PKA-CREB, MAPK/ERK, and PI3K-Akt) that augment BDNF-mediated neurotrophic support. The data collectively substantiate an adaptive neuroprotective mechanism in which orexin enhances receptor signaling and trophic tone to mitigate ROS-induced injury. Together, these findings suggest that HCRT may enhance glial resilience by increasing orexin receptor and BDNF-related transcriptional responses, although future validation in additional cell models and protein-level assays will be needed to confirm the broader neuroprotective relevance of this mechanism.

KEYWORDS: Biology, Cell Biology, Orexin (HCRT), A172 Glial Cells.

■ Introduction

Sleep is a biological activity that is important to the health and well-being of humans.¹ Past research has established that sufficient sleep is important to the proper functioning of the immune system.² This implies the existence of a relationship between lack of sleep and a high potential of developing cardiovascular disease, cancer, and depression.³ Besides its impact on the vulnerability to a disease, sleep promotes necessary physiological functions. This beneficial outcome involves hormone control, repairing tissues, and growth. All these functions are critical to physical and mental health. Thus, sleep research and its processes are important for understanding how the body maintains homeostasis.⁴

Orexin, also known as hypocretin, is a protein that may function as a signal sent throughout the brain.⁵ According to a previous study, the neurons in the lateral hypothalamus release orexin A and B, which are neuropeptides—types of chemical messengers within the brain.⁶ Orexin binds to orexin receptors, such as *OX1R*, to promote systems like the noradrenergic system, the histaminergic system, and the cholinergic system, making orexin crucial in sleep-wake regulation in that it promotes wakefulness.⁷ A previous study also supports this by showing that mice lacking orexin exhibited fragmented wakefulness, suggesting that orexin is key for sustaining wakefulness, not just initiating it.⁸

Chronic sleep deprivation triggers cellular stress responses in the human brain. Previous studies indicated that prolonged wakefulness leads to an increase in reactive oxygen species (ROS) in mouse and human brain cells.⁹ This study indicates that a high level of ROS causes damage to cellular components in mitochondria. When the ROS-induced damage is critical, the mitochondria finally release cytochrome C, triggering apoptosis, a cell suicide mechanism.¹⁰ This apoptosis is activated to eliminate the damaged cells in the human brain. However, eventually these consequences lead to neuronal loss and reduced synaptic integrity.¹⁰

Additionally, sleep deprivation has been shown to lower levels of brain-derived neurotrophic factor (*BDNF*).¹¹ This gene encodes a critical protein for neuronal growth, plasticity, and survival. Therefore, previous studies indicate that lower *BDNF* levels may be a key indicator of brain damage in the human brain. Overall, this information suggests that the combined effects of increased ROS, apoptosis, and diminished neurotrophic support.¹² In addition, these cellular alterations may predispose individuals to neurodegenerative processes. Therefore, this mechanism links chronic sleep disturbance to conditions such as Alzheimer's and Parkinson's disease.¹³ Together, the combined effects of increased ROS, apoptosis, and diminished neurotrophic support may create a cellular environment that increases glial vulnerability and contributes to impaired neuronal support during chronic sleep disturbance.

Existing *in vitro* systems of sleep disruption are weak in their capacity to reflect the complex physiology of hyperarousal and disorganized sleep.¹⁴ The available methods are mostly based on cortisol therapy, which is mostly an expression of overall stress reactions. Therefore, this study establishes a novel glia-centered *in vitro* model for examining how HCRT overexpression influences oxidative stress resilience and neuroprotective gene regulation in A172 glial cells. According to this approach, it is possible to simulate hyperarousal-like states more specifically. The approach is also able to identify glia cells, which are important because they are important factors in neuroinflammation, synaptic support, brain homeostasis, etc., but have been neglected in the literature about sleep disturbance. This model proposes an alternative way to study the glial role in neurophysiological mechanisms that are usually discussed as those of neurons by paying attention to orexin-mediated signaling pathways in glia. Finally, this method can be used to assist in offering more specific simulations of hyperarousal-like states, considering the glial cell. To address this gap, the present study develops a glia-centered *in vitro* model of orexin-associated stress signaling by using lentiviral HCRT overexpression in A172 glial cells. This approach differs from conventional cortisol-based stress paradigms by isolating the potential contribution of orexin signaling to oxidative stress resistance, apoptosis regulation, and BDNF-associated neuroprotective responses."

This study is important because it uses lentivirus-mediated orexin overexpression in human glial cells to model sleep disturbance in a new way. This method is different from traditional models that use generalized stress responses because it focuses on molecular markers that are directly related to sleep regulation, like orexin receptor activity and BDNF expression. By looking at downstream effects like oxidative stress and apoptosis, this study provides a clearer and more mechanistic picture of what happens to cells when sleep is disrupted. This research enhances our understanding of glial involvement in sleep physiology and may facilitate the development of orexin-targeted therapies for insomnia and associated disorders.

■ Methods

Cell Culture:

A172 human glioblastoma glial cells (ATCC CRL-1620; A172 cells in Gibco, USA) were grown in Dulbecco Modified Eagle Medium (DMEM, Gibco, USA) with 10% FBS mixed in and 1% penicillin-streptomycin. Cells were kept at 37 °C in a humidified environment with 5 percent CO₂. All the experiments were done on cells between passages 5 and 15.

Lentiviral-Mediated Orexin Overexpression:

A lentiviral virus plasmid with the human prepro-orexin (HCRT) gene under the control of a CMV promoter was acquired commercially (VectorBuilder). This was done through the production of viral particles in HEK293T packaging cells and harvested at 48-72 h after transfecting the cells. The differentiated A172 cells with retinoic acid were transduced with a multiplicity of infection (MOI) of 20 with 80 µg/mL polybrene. Empty lentivirus was given as a control group. The 2

µg/mL of puromycin was used to select the cells after 72 h to maintain steady expression over a period of 5 days. qPCR was used to identify overexpression efficiency.

Quantitative PCR (qPCR):

The A172 cells were lysed using the Total RNA extraction kit (Bioneer). Quantification of RNA was done by Nanodrop, and 1 µg was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) and run on a QuantStudio 6 system using SYBR Green Master Mix (Applied Biosystems). Primer sets were specifically made against orexin (HCRT), orexin receptor 1 (OX1R), BDNF, and a reference gene, GAPDH. Relative gene expression was calculated using the 2^{-ΔΔCt} method.

Cell Death and Viability Assay:

The use of an automated cell counter (LUNA-FL™, Logos Biosystems) with dual staining of Acridine Orange/Propidium Iodide (AO/PI) was used to measure cell viability and death. Cells were collected, stained, and analysed instantly to identify living, apoptotic, and dead cells. The outcomes were in the form of the percentage of dead cells relative to the total cell count.

Oxidative stress induction and viability/apoptosis analysis:

To evaluate whether HCRT overexpression altered cellular resilience under oxidative stress, A172 cells were first transduced with lentiviral HCRT or a control vector and allowed to recover for 24 h. Oxidative stress was then induced by treating cells with hydrogen peroxide (H₂O₂) at a final concentration of 100 µM for 24 h. Following H₂O₂ exposure, cell viability and apoptosis were assessed. All groups were processed using the same timing after transduction to ensure direct comparison between control and HCRT-overexpressing cells.

Statistical Analysis:

Every experiment was done in triplicate (n = 3). Results are reported in the form of mean standard deviation (SD). One-way ANOVA and *post hoc* test (Tukey) were used to test statistical significance. A p-value of less than 0.05 was taken as a significant p-value.

■ Results and Discussion

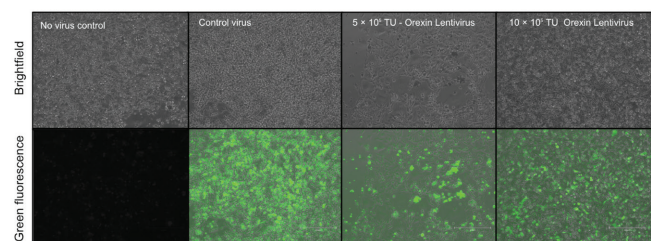


Figure 1: Lentiviral transduction efficiency of Orexin-expressing constructs visualized by GFP fluorescence. Brightfield (top row) and green fluorescence (bottom row) images show A172 cells transduced with increasing doses of Orexin lentivirus. GFP fluorescence increased with viral titer, indicating a clear dose-dependent rise in transduction efficiency. No fluorescence was detected in the no-virus or empty-virus controls, confirming specificity and low background signal. Scale bar = 300 µm.

To evaluate the transduction efficiency of the Orexin-expressing lentivirus, A172 cells were infected with increasing doses of viral particles (5×10^5 and 10×10^5 TU) and examined for GFP fluorescence intensity as a measure of transgene expression. Figure 1 illustrates that both brightfield and green fluorescence images unequivocally demonstrate the successful delivery and expression of lentivirus, exhibiting a dose-dependent efficacy.

The no-virus control and control-virus groups, in contrast, exhibited no detectable fluorescence, thereby confirming the minimal background autofluorescence and the specificity of lentiviral-mediated expression (Figure 1). Cells infected with 5×10^5 TU Orexin lentivirus exhibited moderate GFP signals distributed throughout the culture. In contrast, cells infected with 10×10^5 TU exhibited robust and uniform fluorescence, indicating effective transduction and strong expression of the Orexin gene. Importantly, no cytotoxicity or morphological abnormalities were observed in either infection scenario, indicating the biocompatibility of the lentiviral delivery system.

The rise in GFP fluorescence intensity with higher viral doses shows that the Orexin lentiviral construct works better in human glial-derived A172 cells when the doses are higher. The successful gene delivery achieved with 10×10^5 TU demonstrates that the lentiviral system enabled stable integration and sustained expression without notable cytotoxic effects.

This finding validates a reliable *in vitro* model for subsequent molecular and functional assays investigating Orexin-related neuroprotective signaling. The absence of background signal in the control conditions ensures that GFP expression is exclusively a result of transgene delivery, rather than nonspecific fluorescence or viral artifacts.

These results confirm that lentivirus-mediated overexpression is a viable method for modulating Orexin signaling in neuronal and glial cell models. The established expression gradient (5×10^5 vs. 10×10^5 TU) serves as a basis for subsequent analyses, including qPCR quantification of *HCRT*, *OX1R*, and *BDNF*, to clarify dose-dependent effects on neurotrophic and neuroprotective pathways related to sleep regulation and stress adaptation.

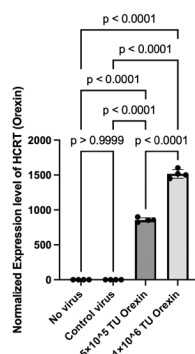


Figure 2: Quantitative PCR analysis of *HCRT* (Orexin) mRNA expression following lentiviral transduction. RT-qPCR measured relative mRNA expression levels of *HCRT* in A172 cells transduced with increasing doses of Orexin lentivirus (5×10^5 and 1×10^6 TU), compared to no-virus and control-virus groups. *HCRT* mRNA was robustly and titratably overexpressed, rising from baseline in controls to $\sim 8.6 \times 10^2$ -fold (5×10^5 TU) and $\sim 1.5 \times 10^3$ -fold (1×10^6 TU). This confirms successful, dose-dependent orexin induction at the transcript level ($n=3$; $p<0.0001$ vs. controls). Data are presented as mean $\pm$ SD ($n=3$).

Lentiviral transduction of A172 cells produced a strong, dose-dependent increase in *HCRT* (Orexin) mRNA (Figure 2). Relative to the control-virus calibrator (set to 1.0), the 5×10^5 TU Orexin group reached $\sim 8.6 \times 10^2$ -fold expression (mean $\pm$ SD: 857 ± 40), and the 1×10^6 TU group reached $\sim 1.5 \times 10^3$ -fold (1517 ± 76), while no-virus and control-virus groups remained at baseline (≈ 0.8 – $1.0 \times$). One-way ANOVA with Tukey's multiple comparisons showed that both Orexin groups were significantly higher than either control ($p < 0.0001$ for all indicated comparisons; $n=3$). These data confirm successful and titratable overexpression of Orexin at the transcript level.

The RT-qPCR results back up the fluorescence results. This data shows that lentiviral delivery results in strong, dose-dependent Orexin expression, whereas controls show no noticeable increase. It is clear that increasing viral input from 5×10^5 to 1×10^6 TU, about twice as much, results in more *HCRT* mRNA. This indicates that expression has not yet reached its maximum in this range. This establishes a reliable model for overexpression that facilitates the investigation of downstream signaling.

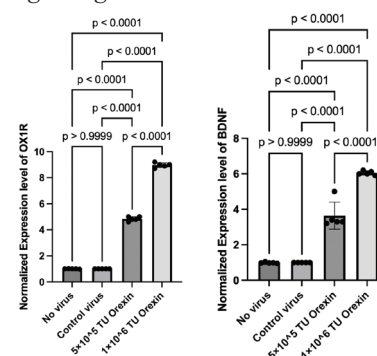


Figure 3: Orexin overexpression upregulates *OX1R* and *BDNF* gene expression in A172 cells. Quantitative RT-qPCR analysis of *OX1R* (left) and *BDNF* (right) mRNA levels following lentiviral transduction of Orexin in A172 cells. Data are presented as mean $\pm$ SD ($n=3$). Orexin overexpression coordinately increased both *OX1R* and *BDNF*, consistent with strengthened receptor signaling and elevated neurotrophic tone. The higher viral dose produced the largest induction (*OX1R* ~ 9 -fold; *BDNF* ~ 6 -fold), supporting a dose-dependent pro-survival molecular response ($n=3$; $p<0.0001$). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test.

Orexin overexpression produced a dose-dependent induction of both *OX1R* and *BDNF* mRNA in A172 cells (Figure 3). Relative to the control-virus calibrator (set to 1.0), the 5×10^5 TU Orexin group showed a ~ 4.8 -fold increase in *OX1R* (mean $\pm$ SD: 4.8 ± 0.2) and a ~ 3.3 -fold increase in *BDNF* (3.3 ± 0.1). The 1×10^6 TU Orexin group further increased expression to ~ 9.0 -fold for *OX1R* (9.0 ± 0.15) and ~ 6.0 -fold for *BDNF* (6.0 ± 0.15). No-virus and control-virus groups remained at baseline. One-way ANOVA with Tukey's multiple comparisons indicated that both Orexin groups were significantly higher than either control for both genes ($p < 0.0001$, all indicated comparisons; $n=3$).

The results show that overexpression of Orexin increases *OX1R* signaling and *BDNF* expression. This supports the idea of an adaptive neuroprotective mechanism in the sleep disturbance model. In the field of sleep-related neurobiology,

elevated Orexin levels are expected to enhance Orexin receptor signaling and activity-dependent trophic pathways. Figure 3 illustrates the simultaneous upregulation of both *OX1R* and *BDNF*. This supports a functional model in which Orexin overexpression can trigger adaptive, neuroprotective mechanisms essential for stress or sleep disturbance conditions. This result shows the numbers needed to link viral dose to molecular readouts. We can then use this system to perform functional tests, such as assessing how well it performs under oxidative stress.

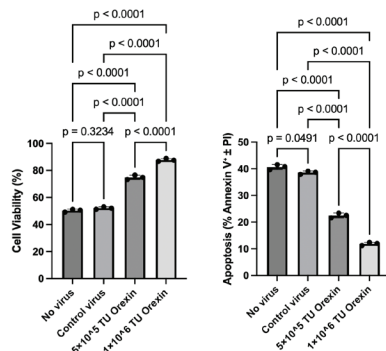


Figure 4: Orexin overexpression confers resistance to oxidative stress. A172 cells transduced with Orexin lentivirus exhibited significantly higher viability and lower apoptosis after H_2O_2 exposure than the no-virus and control-virus groups. Under oxidative stress, orexin significantly improved survival and reduced apoptosis in a dose-dependent manner, demonstrating functional cytoprotection. These effects align with the observed *OX1R*/*BDNF* upregulation, supporting an orexin-linked adaptive neuroprotective axis against ROS-induced injury (n=3; p<0.0001 vs. controls).

Orexin overexpression significantly protected A172 cells from H_2O_2 -induced damage (Figure 4). In the viability assay, means ($\pm$ SD, n=3) rose from ~50–52% in no-virus and control-virus groups to ~75% at 5×10^5 TU and ~88% at 1×10^6 TU (one-way ANOVA with Tukey's test, p < 0.0001 for all Orexin vs. control comparisons; no-virus vs. control-virus not significant, p = 0.3234). In the apoptosis assay (Annexin V⁺ $\pm$ PI), means declined from ~40–41% (no-virus/control-virus) to ~22% at 5×10^5 TU and ~12% at 1×10^6 TU (p < 0.0001 for Orexin vs. controls; a small difference between no-virus and control-virus, p = 0.0491). Together, these data show a clear dose-dependent cytoprotective effect of Orexin.

Given consistent transcriptional increases in *HCRT*, *OX1R*, and *BDNF*, Orexin overexpression may provide neuroprotection against oxidative stress. This may enhance survival and reduce apoptosis in A172 cells. This result indicates that stronger *OX1R* signaling may involve the cAMP/PKA-CREB, MAPK/ERK, and PI3K-Akt pathways, which can activate *BDNF* and other protective programs that prevent cell death. This mechanism protects them from H_2O_2 -induced damage.

Although this study did not directly manipulate sleep duration, *HCRT* overexpression was used to model a key molecular feature of sleep disturbance: heightened orexinergic/hypocretin signaling associated with wakefulness, arousal, and stress-related hyperarousal. Orexin neurons promote sustained wakefulness and activate arousal systems; therefore, increasing *HCRT* expression in glial cells provides a reductionist *in vitro* approach for examining how an orexin-enriched cellular envi-

ronment may influence glial resilience under oxidative stress. This model does not reproduce the full systemic physiology of sleep deprivation, but it offers a controlled platform to isolate the relationship between *HCRT* signaling, oxidative stress response, and neurotrophic gene regulation.

Study impact:

This study presents a focused, glia-centric model of sleep disturbance that isolates orexin-driven biology and correlates it with quantifiable cellular protection, enhancing both mechanism and methodology. We demonstrate that lentiviral orexin overexpression enhances *OX1R* and *BDNF* levels while mitigating oxidative-stress injury, thereby establishing a coherent, adaptive neuroprotective axis that is amenable to direct testing. The model serves as a functional platform for evaluating orexin-pathway modulators and *BDNF*/TrkB enhancers, establishing benchmarks for biomarkers of hyperarousal and neuroprotection, and analyzing glial contributions to sleep-related pathology—an aspect frequently neglected in neuron-centric research.¹⁵ Translationally, the results emphasize orexin-targeted interventions as potential treatments for insomnia and stress-related neurodegeneration, and they describe pharmacologic experiments (*OX1R* antagonism and *BDNF* blockade) that can reduce the risk of therapeutic strategies.¹⁶ In general, this work makes things clearer on a mechanistic level and provides a scalable assay framework that speeds up the process of going from cellular insight to therapy design.

Limitations and Future Study:

This work has several limitations that should be addressed in follow-up studies. First, results were derived from an immortalized glial line (A172), which may not recapitulate primary human glia or neuron-glia interactions; future experiments should validate findings in primary astrocytes/microglia, human iPSC-derived co-cultures, brain organoids, and *in vivo* models. Second, the lentiviral overexpression paradigm may exceed physiological orexin levels and introduce MOI/polybrene or integration-site confounds. Therefore, titration to near-physiological expression, CRISPRa/inducible systems, and isogenic controls are warranted. Third, conclusions rely largely on mRNA. In future studies, protein-level and signaling readouts (*OX1R*/*BDNF* protein, CREB-P, ERK, Akt), secreted *BDNF* ELISA, and receptor function assays should be added. Fourth, causal links should be tested using pharmacology and genetics (*OX1R* antagonists such as SB-334867, TrkB blockade with ANA-12, and siRNA/shRNA knock-down of *HCRT*/*OX1R*/*BDNF*). Fourth, the stress paradigm was limited to H_2O_2 ; expanding to diverse stressors (ER/mitochondrial stress, glutamate excitotoxicity), time-course and single-cell analyses (ROS, $\Delta\Psi_m$, caspase-3/7, scRNA-seq/proteomics), and electrophysiology to assess network effects will clarify the breadth, mechanism, and translational relevance of the proposed neuroprotective axis. Finally, the present study evaluates *OX1R* and *BDNF* primarily at the mRNA level using RT-qPCR. Because transcript abundance does not always predict protein abundance, the results should be interpreted as

evidence of transcriptional regulation rather than direct confirmation of receptor or neurotrophic protein changes. Future studies should include Western blotting or ELISA to determine whether HCRT-induced increases in OX1R and BDNF mRNA are accompanied by corresponding protein-level changes and to strengthen the proposed neuroprotective mechanism.

■ Conclusion

The study was successfully able to establish a glial-cell model using the lentivirus-mediated Orexin (HCRT) overexpression to study its reaction to stress conditions. Resultantly, this model showed increased Orexin, OX1R, and BDNF expression. In addition, it was observed that cell viability increased while apoptosis decreased under oxidative stress, showing increased cell survival and resilience.

Such molecular and functional outcomes suggest Orexin activates neuroprotective signaling pathways for increased resilience under stress. Specifically, Orexin likely engages OX1R-dependent cascades, such as cAMP/PKA-CREB, MAPK/ERK, and PI3K-Akt, all of which are pathways that support the plasticity of cells. In our observation, activation of these pathways led to BDNF-mediated neuronal survival and stress resistance.

Such mechanisms suggest that orexin signaling represents an adaptive response to sleep disturbance or oxidative injury. By increasing cell viability, reducing apoptosis, and expression of BDNF, a crucial factor for survival, Orexin appears to have a role in maintaining integrity under stress conditions such as sleep deprivation or hyperarousal. In conclusion, lentivirus-mediated Orexin overexpression offers a reliable platform to study cellular mechanisms underlying sleep disturbance. It also underscores the potential for Orexin-targeted pathways to be used as therapeutic avenues for insomnia and neurodegenerative conditions due to Orexin's role in enhancing neuronal survival and stress resilience.

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