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Orexin A decreases lipid peroxidation and apoptosis in a novel hypothalamic cell model

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HIGHLIGHTS

- Orexin A (hypocretin 1) increases neuroprotection in a hypothalamic cell line.
- Neuroprotection is partly due to decreases in caspase 3/7 and lipid peroxidation.
- Suggests mechanism through which orexin could protect against high-fat diet.

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ABSTRACT

Current data support the idea that hypothalamic neuropeptide orexin A (OxA; hypocretin 1) mediates resistance to high fat diet-induced obesity. We previously demonstrated that OxA elevates spontaneous physical activity (SPA), that rodents with high SPA have higher endogenous orexin sensitivity, and that OxA-induced SPA contributes to obesity resistance in rodents. Recent reports show that OxA can confer neuroprotection against ischemic damage, and may decrease lipid peroxidation. This is noteworthy as independent lines of evidence indicate that diets high in saturated fats can decrease SPA, increase hypothalamic apoptosis, and lead to obesity. Together data suggest OxA may protect against obesity both by inducing SPA and by modulation of anti-apoptotic mechanisms. While OxA effects on SPA are well characterized, little is known about the short- and long-term effects of hypothalamic OxA signaling on intracellular neuronal metabolic status, or the physiological relevance of such signaling to SPA. To address this issue, we evaluated the neuroprotective effects of OxA in a novel immortalized primary embryonic rat hypothalamic cell line. We demonstrate for the first time that OxA increases cell viability during hydrogen peroxide challenge, decreases hydrogen peroxide-induced lipid peroxidative stress, and decreases caspase 3/7 induced apoptosis in an *in vitro* hypothalamic model. Our data support the hypothesis that OxA may promote obesity resistance both by increasing SPA, and by influencing survival of OxA-responsive hypothalamic neurons. Further identification of the individual mediators of the anti-apoptotic and peroxidative effects of OxA on target neurons could lead to therapies designed to maintain elevated SPA and increase obesity resistance.

1. Introduction

The orexins (hypocretins) are hypothalamic neuropeptides (orexins A and B; also known as hypocretins-1 and -2) that act on two related G-coupled protein receptors (OxR1 and OxR2) to influence diverse physiological processes such as control of food intake, sleep–wake behavior, arousal, energy balance, and energy expenditure [12,19,32,37]. The most immediate cellular response to orexin receptor activation in both overexpression and *in vivo* models is increased intracellular Ca^{2+} influx, by either protein kinase C-dependent activation or by voltage-gated Ca^{2+} receptors [2,3]. Additional downstream pathways of activated orexin receptors include the kinase activity of extracellular-signal-regulated kinases (ERK1/2) and p38 mitogen activated phosphate kinase

Abbreviations: ERK1/2, extracellular-signal-regulated kinases 1 and 2; HEK, human embryonic kidney; HFD, high fat diet; HIF-1α, hypoxia-inducible factor 1 alpha; p38 MAPK, p38 mitogen activated phosphate kinase; MDA, malondialdehyde; NEAT, non-exercise activity thermogenesis; OR, obesity resistance; OxA, orexin A/hypocretin 1; OxR1, orexin/hypocretin receptor 1; OxR2, orexin/hypocretin receptor 2; POMC, proopiomelanocortin; RFU, relative fluorescent units; rLH, rostral lateral hypothalamus; RLU, relative luminance units; RT-PCR, real-time reverse transcriptase polymerase chain reaction; SPA, spontaneous physical activity; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

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(MAPK) [3,36]. The degree of orexin-induced pathway activation at either receptor appears to be influenced by cell type and specific G-α subunit proteins receptor coupling [3,18].

The biological control of physical activity is relatively unexplored [25], but is important to body weight regulation. Activities outside of volitional exercise are known as spontaneous physical activity (SPA), which generates non-exercise activity thermogenesis (NEAT) [23,44]. Data suggest that NEAT can vary by as much as 2000 kcal/day between individuals [26], and that NEAT and SPA levels are predictive of weight gain and obesity resistance (OR) in humans [24] and rodents [19]. SPA effects on OR and weight gain depend in part upon OxA signaling in the rostral lateral hypothalamus (rLH) [44]. OxA promotes SPA when injected into rLH, and rats with higher OxA responsiveness have higher gene expression levels for OxA receptors [32,42,44]. At present the intracellular signaling mechanisms through which OxA might mediate short- or long-term changes in SPA and OR are unknown.

Given the multiple physiological processes and second messenger pathways it activates, OxA potentially has pleiotropic effects. OxA alters intracellular metabolic function and cell survival in neuronal tissue [15,39,40,49]. OxA-induced neuroprotective mechanisms are due in part to increases in HIF-1α and decreased oxidative stress [15,39,49]. OxA increases ATP *via* induction of the transcription factor hypoxia inducible factor-1α (HIF-1α) in mouse hypothalamic tissue under normoxic conditions [39]. In ischemic conditions, OxA promotes the survival of primary cortical neurons *in vitro*, and suppresses neuronal damage by regulating post-ischemic glucose intolerance *in vivo* [15,39,40,49]. No published studies have evaluated the potential neuroprotective effects of OxA in the hypothalamus.

Mounting data suggest that diets high in saturated fats may induce hypothalamic neurodegenerative pathways [29,45] and contribute to several disease processes including obesity. High fat diets (HFDs) are linked to obesity and increases in lipid peroxidative metabolites, induction of oxidative stress, neurodegeneration, and subsequent changes in neural plasticity [14,48]. Broader hypothalamic neurodegeneration through apoptotic mechanisms induced by HFD consumption and increased oxidative stress has been shown [45,46]. HFD can promote obesity purely through changes in energy balance, but findings of hypothalamic inflammation, neurodegeneration, and apoptosis imply additional critical factors that have not been fully explored.

It is unclear if these disturbances alter hypothalamic control of SPA, but HFD (particularly high saturated fat diet) increases brain oxidative stress and inflammation in rats [5,51], whereas moderate physical activity reduces CNS oxidative stress [31]. While OxA signaling promotes SPA [21,22,44], and increased SPA protects against obesity [43,44], whether OxA protects against HFD-induced oxidative stress in the hypothalamus is unknown. Multiple models link changes in OxA or orexin receptors to neurodegenerative conditions [13,17], suggesting that orexin signaling pathways help maintain neuronal survival. Increased survival of neurons responsible for mediating SPA might explain the long-term benefits of OxA in obesity resistance, but how OXA-induced intracellular mechanisms affect short-term neuronal survival or SPA response is poorly understood.

The lack of appropriate cell lines has proven an obstacle to modeling neuroendocrine mechanisms such as those underlying the pleiotropic effects of OxA. The recent development of immortalized hypothalamic cell lines provides an opportunity for preliminary evaluation of cellular signaling mechanisms without the complexity of the intact architecture of the hypothalamus. In this study a novel clonal immortalized embryonic rat hypothalamic cell line was used to evaluate whether OxA protects against oxidative stress-induced apoptosis [4,28]. Experiments were performed to test whether pretreatment with OxA could increase cell survival, reduce apoptotic caspase activity, and reduce lipid oxidative damage in hypothalamic cells during an oxidative stress challenge using the pro-oxidant hydrogen peroxide (H_2O_2).

2. Methods

2.1. Cell culture and reagents

Immortalized embryonic rat hypothalamic cells (R7; CELLutions-Cedarlane, NC, USA) were used for all studies. R7 clonal cells were derived from E17 rat hypothalamic primary cultures, immortalized by retroviral transfer of simian virus of T antigen, and subcloned to homogeneity [4,28]. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37 °C with 5% CO_2 and plated at 25,000 per well in 96 well plates. OxA peptide (American Peptides, Sunnyvale, CA, USA) was suspended in artificial cerebrospinal fluid and diluted to final concentrations of 50, 100, or 300 nM in DMEM. Hydrogen peroxide (Sigma, St. Louis, MO, USA) was diluted to final concentration (50 μM) in DMEM as previously described [47].

For all assays, cells were incubated with DMEM/vehicle, OxA alone, H_2O_2 alone, or H_2O_2 plus OxA. Manufacturer protocols were followed for all kits used, and for all assays SpectraMax-M5 reader (Molecular Probes, Sunnyvale, CA, USA) was used to determine relative fluorescence (RFU) or luminance (RLU).

Real-time RT-PCR: Real-time RT-PCR for Ox1R and Ox2R was performed using 200–250 ng of total RNA from Sprague Dawley rat lateral hypothalamus or R7 cells. Extraction methods, PCR primers, and methodology have been previously described [10,20,44]. Amplification products were visualized by electrophoresis using a 2% agarose gel stained with SYBR green (Invitrogen, Grand Island, NY, USA).

Western blots: Orexin receptor protein levels were assayed using 50 μg of R7 cell, rat whole hypothalamic tissue (positive control), and human embryonic kidney (HEK) cell (negative control [41]) lysate. Western blots were prepared as previously described [7]. Primary antibodies for Ox1R (AB3092; Millipore, Billerica, MA, USA) and Ox2R (H00003062-Q01; Novus Biologicals, Littleton, CO, USA) were used at a final dilution of 1:1000 and visualized using HRP-conjugated secondary antibodies (Novus) [7].

Calcium assay: Change in intracellular Ca^{2+} concentration following incubation with OxA was measured using the Fluo-4 Direct Calcium Assay Kit (Invitrogen).

Viability assay: Cell viability was determined using a resazurin-based assay (Invitrogen). Data are reported as percent RFU change *vs.* control.

Caspase activity: Caspase 3/7 activity was determined by the addition of a DEVD substrate (Promega, Madison, WI, USA). Data are reported as percent RLU change *vs.* control.

Thiobarbituric acid reactive substances (TBARSs) assay: Lipid peroxidation was measured by determining the generation of malondialdehyde (MDA; Cayman, Ann Arbor, MI, USA) as previously described [11].

Statistical methods: Significant differences were determined by unpaired, two-tailed *t*-test using GraphPad Prism 5 (GraphPad, San Diego, CA, USA).

3. Results

Real-time RT-PCR shows that rat R7 cells express orexin receptor mRNA (Fig 1A), and Western blot data show that this mRNA is translated into mature receptor protein (Fig. 1B). Receptor function was determined using calcium assay, as the binding of OxA

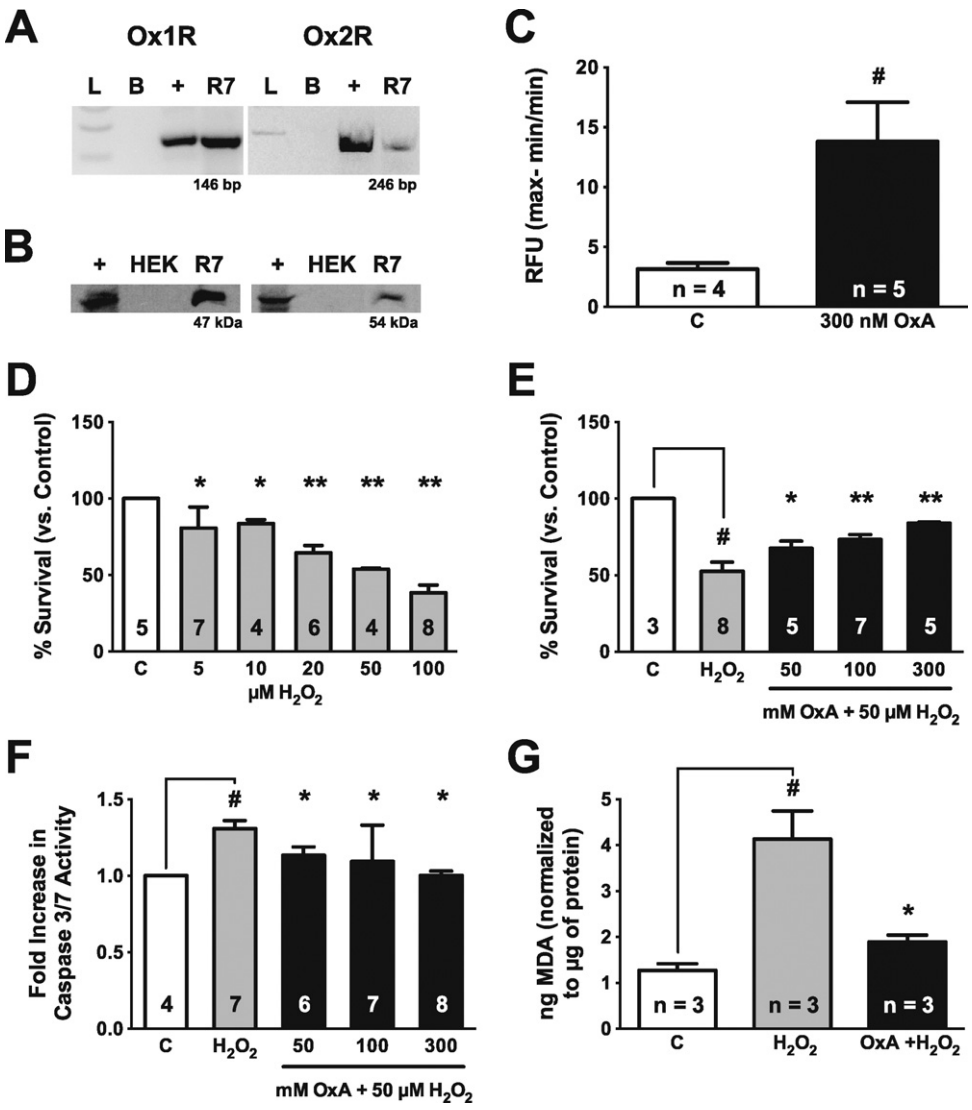

Fig. 1. (A–C) Rat R7 cells endogenously express functional orexin receptors. (A) Real time RT-PCR demonstrating expression of Ox1R and Ox2R mRNA. L, ladder; B, blank; +, rat lateral hypothalamus positive control; R7, rat R7 cell line. (B) Western blot showing Ox1R and Ox2R receptor protein. +, rat hypothalamic lysate; HEK, human embryonic kidney cell (negative control); R7, rat R7 cell lysate. (C) Calcium assay showing orexin receptors are functional. R7 neurons treated with 300 nM OxA significantly increased intracellular Ca^{2+} relative to control ($p = 0.0249$). (D–G) Orexin A pretreatment attenuates H_2O_2-induced cell death, apoptosis, and lipid peroxidation in R7 cells. (D) 24 h H_2O_2 treatment significantly and dose-dependently decreased cell viability (fold change in RFU). (E) Pretreatment with OxA significantly and dose-dependently reversed effects of H_2O_2 challenge. Neurons were pretreated with OxA (50, 100 or 300 nM) for 24 h and then challenged with 50 μM H_2O_2. Additional dose of OxA was given at the time of H_2O_2 challenge. Viability was assayed 24 h post-challenge to determine the percentage of living cells. (F) 1 h challenge with 50 μM H_2O_2 significantly increased caspase 3/7 activity (fold increase in RLU), and 24 h pretreatment with OxA significantly decreased caspase 3/7 activity in H_2O_2 challenged cells. (G) H_2O_2 challenged cells showed significantly increased lipid peroxidation (determined by the generation of malondialdehyde (MDA) using the TBARS assay), and OxA pretreatment significantly reduced effects of H_2O_2. Data in C expressed as relative fluorescent units (RFUs); data in D–G normalized relative to controls. Group sizes indicated by numbers on columns. $^{\#}p < 0.05$ *vs.* control; $^{*}p < 0.05$ *vs.* H_2O_2; $^{**}p < 0.0001$ *vs.* H_2O_2.

to Ox1R or Ox2R is associated with a robust increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) [16]. R7 cells incubated with 300 nM OxA showed an immediate significant increase in $[Ca^{2+}]_i$ ($p = 0.0249$; Fig. 1C). Together these results suggest that R7 cells are a suitable *in vitro* model for studying the potential pleiotropic effects of OxA.

To study the effect of OxA during oxidative stress, we used H_2O_2, which induces neuronal apoptosis at low concentrations (Fig. 1D) [1,47]. To determine if OxA can prevent H_2O_2-induced cell death and apoptosis, cells were pretreated for 24 h with different concentrations of OxA (either 0, 50, 100, or 300 nM). Following pretreatment with OxA, cells were then challenged with 50 μM H_2O_2 for 24 h in the presence of a second dose of 0, 50, 100, or 300 nM OxA. Viability in H_2O_2-treated cells was significantly reduced ($p < 0.0001$), and pretreatment with OxA significantly increased viability at all doses (50 nM: $p = 0.0009$; 100, 300 nM: $p < 0.0001$), indicating that OxA pretreatment was neuroprotective in a dose dependent manner (Fig. 1E).

To test whether OXA pretreatment decreased apoptosis, cells were pretreated with OxA using the doses and time course described above, and caspase 3/7 activity was assayed following a 3 h exposure to 50 μM H_2O_2. Duration of treatment was based on a prior study examining H_2O_2-induced apoptosis in primary mouse neuronal culture [47]. Consistent with this previous study, H_2O_2 treatment significantly increased caspase 3/7 activity relative to controls ($p = 0.0075$; Fig. 1F). Remarkably, at all doses tested, OxA pretreatment significantly decreased caspase activity relative to H_2O_2-treated cells without pretreatment (50 nM: $p = 0.0137$; 100 nM: $p = 0.0367$; 300 nM: $p = 0.0108$).

OxA has previously been shown to decrease oxidative cell death in mouse cerebral cortical tissue [15]. To determine whether a similar mechanism may exist in hypothalamic cells, we tested

if OxA could decrease lipid peroxidation during H_2O_2 challenge. Cells were pretreated with 300 nM OxA for 24 h and then challenged with 50 nM H_2O_2 for 24 h as described above. Samples were normalized to μg of protein and reported as ng MDA. H_2O_2 challenge significantly increased lipid peroxidation relative to controls ($p = 0.0103$; Fig. 1G), and OxA pretreatment significantly decreased lipid peroxidation relative to non-pretreated H_2O_2-treated cells ($p = 0.0236$).

4. Discussion

These data indicate that the novel rat R7 hypothalamic cell line represents a suitable *in vitro* model for evaluation of OxA-induced changes in cell survival following oxidative stress. A major advantage of the R7 line is derivation from differentiated primary hypothalamic neurons. The resulting cells likely exhibit characteristics more typical of normal hypothalamic cells, and derived cell line data thus more likely represent the potential neuroprotective role of OxA *in vivo*.

R7 cells express functional orexin receptors (as shown by RT-PCR, Western blot, and increased $[Ca^{2+}]_i$ following OxA treatment; Fig. 1A–C). Consistent with prior publications, H_2O_2 challenge in R7 cells decreased cell viability (Fig. 1D–E), increased caspase activity (Fig. 1F), and increased lipid peroxidative damage (Fig. 1G). In all cases, OxA pretreatment significantly reversed H_2O_2-induced damage. These data indicate that OxA neuroprotection may involve decreased onset of caspase-committed apoptosis. This is the first time that OxA has been shown to decrease apoptosis in hypothalamic tissue. Likewise, while OxA has previously been reported to decrease ischemia-induced peroxidative damage in cortex and gastric tissues *in vitro* and *in vivo* [6,15,49], this is the first report that OxA can reduce peroxidative damage in hypothalamic cells. Given that hypothalamic regions receive greater innervation by orexin fibers than does cortex [33], OxA-mediated neuroprotective mechanisms established in cortex may be even more important in the hypothalamus.

Little is known about the short- and long-term effects of OxA signaling on intracellular neuronal metabolic status or the physiological relevance of this signaling to SPA. Collectively, emerging evidence indicates that activation of Ox1R/Ox2R by OxA alters proteins involved in intracellular metabolic function [39]. Recently, OxA has been shown to increase ATP and hypoxia inducible factor-1α (HIF-1α) in hypothalamic tissue under normoxic conditions [39]. This is noteworthy given that HIF-1α increases oxidative phosphorylation. Additional independent studies have shown that HIF-1α expression is regulated in part by mitogen-activated protein kinases and the transcriptional coactivator PGC-1α [9,34,38]. PGC-1α is a regulator of mitochondrial biogenesis, and can simultaneously upregulate genes that protect against oxidative stress, while increasing ATP production [27]. The specific function of PGC-1α in brain and neuronal metabolism is still under investigation, and there is increasing interest in its role in neuronal survival and systemic energy balance [35]. A recent study showed that mice lacking functional HIF-1α and HIF-2α proteins in hypothalamic pro-opiomelanocortin (POMC) neurons (POMC/HIFβ mice) have impaired energy expenditure, hyperphagia, and increased fat mass [50]. In the same study, viral overexpression of HIF-1α in the mediobasal hypothalamus resulted in obesity resistance during HFD feeding. Mediobasal hypothalamic neuropeptide Y and POMC neurons are directly responsive to OxA [30], and OxA is known to increase HIF-1α expression in target neurons, suggesting at least one direct connection between OxA signaling and intracellular mechanisms that may influence individual resistance to obesity.

Collectively the data here and previously published support the idea that OxA actions on responsive neurons result not only in promotion of short-term behavioral activity outputs such as SPA, but may also trigger pleiotropic cell signaling effects important in mediating long-term changes in OxA responsiveness and cell survival [8]. As earlier work suggests [32,42], OxA integrates signals that elevate SPA, and these data provide an additional framework in which OxA alters genes that increase intracellular metabolic resistance to HFD-induced oxidative stress. Put into context physiologically, OxA could stimulate neuroprotective mechanisms in OxA-responsive rLH neurons. Increased survival of these SPA-promoting rLH neurons could help individuals resist the damage caused by HFD feeding, conferring resistance to obesity by allowing maintenance of a higher level of both OxA responsiveness and OxA-induced SPA. Conversely, individuals with poor orexin responsiveness might be more susceptible to HFD-induced neurodegeneration, damaging SPA-inducing rLH neurons, leading to obesity. This argument appears to be consistent with previous publications showing that rats resistant to HFD-induced obesity have higher endogenous expression of orexin receptors and greater responsiveness to orexin than do obesity-prone rodents [32,42,44]. Future studies evaluating OxA-induced changes in gene expression and regulation of important second messenger pathways in intracellular neuronal metabolism will elucidate the impact of OxA signaling on long-term energy expenditure changes and weight gain propensity. Understanding the long-term metabolic effects of OxA on hypothalamic target neurons could lead to therapies designed to maintain elevated SPA and increase obesity resistance.

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