



## 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.

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**Abbreviations:** ERK1/2, extracellular-signal-regulated kinases 1 and 2; HEK, human embryonic kidney; HFD, high fat diet; HIF-1 $\alpha$ , 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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### 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<sup>2+</sup> influx, by either protein kinase C-dependent activation or by voltage-gated Ca<sup>2+</sup> 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

(MAPK) [3,36]. The degree of orexin-induced pathway activation at either receptor appears to be influenced by cell type and specific G- $\alpha$  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-1 $\alpha$  and decreased oxidative stress [15,39,49]. OxA increases ATP *via* induction of the transcription factor hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) 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<sub>2</sub>O<sub>2</sub>).

## 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<sub>2</sub> 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  $\mu$ M) in DMEM as previously described [47].

For all assays, cells were incubated with DMEM/vehicle, OxA alone, H<sub>2</sub>O<sub>2</sub> alone, or H<sub>2</sub>O<sub>2</sub> 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  $\mu$ g 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<sup>2+</sup> 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

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