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Effect of Orexin A on Neuropeptide Y Expression in a Novel Immortalized Hypothalamic Cell Line

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Introduction

In rodents, stimulation of food intake by the peptide hormone orexin A (OXA; hypocretin 1) depends in part on neuropeptide Y (NPY) signaling in the hypothalamic arcuate nucleus (Arc) [1]. Arcuate NPY cells express orexin receptors [2, 3], and orexin neurons form synapses on Arc NPY neurons [4]. OXA increases the activity of Arc NPY neurons *in vitro* [2, 5], and OXA stimulates increases in NPY mRNA 2 h post-treatment *in vivo* [6].

Arcuate NPY neurons are also regulated by leptin. Arc NPY neurons express leptin receptor (LepR) [7]. In contrast to orexin, leptin inhibits activity of NPY cells [2] and NPY mRNA expression [8]. While orexin and leptin have apparently inverse effects on Arc NPY neurons, the specific mechanisms through which leptin and orexin signaling pathways might interact to exert short- and long-term control of NPY expression and release is not fully understood.

While a prior *in vitro* model has been proposed using an NPY- and LepR-expressing cell line [9], leptin effects on NPY in this line conflict with *in vivo* results, and interpretation is limited as the cell line used was derived from a human neuroblastoma rather than a normal neuron. We present here preliminary data on an Arc-like model using a mouse cell line derived from immortalized embryonic hypothalamic neurons via retroviral transfer [10]. As this cell line is derived from normal neurons rather than from a tumor, it is more likely to reflect responses of differentiated neurons in vivo [10]. Our initial investigations were designed to (1) validate this cell line as Arc-like, and (2) to begin to investigate mechanisms of OXA action in these cells.

Methods

Cell Line and Maintenance: Differentiated immortalized embryonic mouse hypothalamic cells (designated Clu 121; **Fig. 1**) were purchased from CELLutions-Cedarlane (North Carolina, USA). Cells were maintained in DMEM medium supplemented with 10% FBS at 37°C with 5% CO_2.

Experimental Treatment: OXA peptide (American Peptides, Sunnyvale, CA) was dissolved in PBS and stored at -20°C until the day it was used. Final concentrations were diluted in DMEM. Cells were incubated for 1, 2, 6, or 24 h in either DMEM or DMEM with 300 nanomolar OXA.

Real-time RT-PCR: Total mRNA from cultured cells was isolated using a commercial extraction kit (Qiagen. Isolated mRNA was measured by real-time RT-PCR using a Roche LightCycler. Primers used are summarized in **Table 1**. Relative mRNA levels were normalized to GAPDH using the Δ-ΔCT method.

Figure 1: Clu 121 Hypothalamic Cells

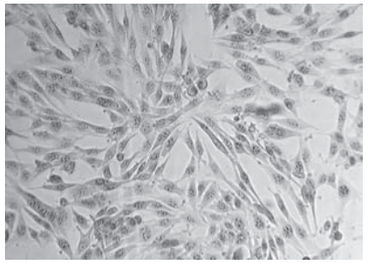


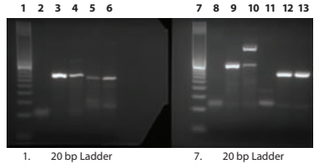
Figure 1: Photomicrograph of Clu 121 cells in culture.

Table 1: Genes, Accession Numbers, and Primers for RT-PCR

Gene	Accession #	Primer Pair		Product Size
Neuropeptide Y	NM_023456	5′–GGACTGACCCTCGCTCTATC–3′ 5′–AGTGTCGCAGAGCGGAGTAG–3′	Forward Reverse	133 bp
Leptin Receptor (Long form)	NM_146146	5′–GTCTCCCTTCTTTTGACCACACC–3′ 5′–CTGACACTCATCCTCACAGGTTACC–3′	Forward Reverse	123 bp
Hypocretin/Orexin Receptor 1	NM_198959	5′–GCGATTATCTCTACCCGAAGC–3′ 5′–CAGGGACAGGTTGACAATG–3′	Forward Reverse	161 bp
Hypocretin/Orexin Receptor 2	NM_198962	5′–AATCCCACGGACTATGACGACG–3′ 5′–GAGAGCCACAACGAACACGATG–3′	Forward Reverse	120 bp
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_008084	5′–GACATCAAGAAGGTGGTGAAGCAG–3′ 5′–AAGGTGGAAGAGTGGGAGTTGC–3′	Forward Reverse	116 bp

Table 1: Genes assayed in Clu 121 cells using real-time RT-PCR. Accession numbers, PCR primer pairs and size of expected products are listed for each gene.

Figure 2: Clu 121 qRT-PCR Gel



1. 20 bp Ladder
2. Blank
3. NPY Control*
4. Clu 121 NPY
5. LepR Control
6. Clu 121 LepR
7. 20 bp Ladder
8. Blank
9. OX1R Control
10. Clu 121 OX1R
11. Blank
12. OX2R Control
13. Clu 121 OX2R

*Controls = C57 Black 6 mouse whole brain mRNA

Figure 2: Gel showing qRT-PCR products for genes assayed. Mouse whole brain mRNA was used for positive controls.

Figure 3: Neuropeptide Y mRNA

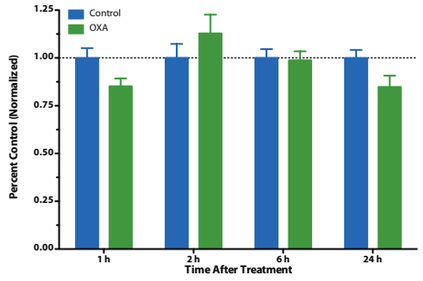


Figure 3: Effect of 300 nM OXA treatment on NPY expression at 1 h, 2 h, 6 h and 24 h. Trend for increased NPY mRNA at 2 h post-treatment.

Figure 4: Leptin Receptor mRNA

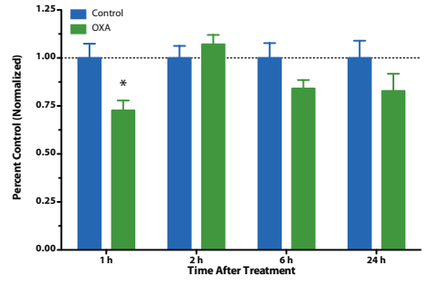


Figure 6: Effect of 300 nM OXA treatment on LepR expression in Clu 121 cells at 1 h, 2 h, 6 h and 24 h. LepR mRNA significantly reduced 1 h post-treatment ($p = 0.014$).

Results

Gene expression for NPY, leptin receptor, and orexin receptors 1 and 2 was verified using real-time RT-PCR (**Fig. 2**) and is consistent with the cell line profile provided by the supplier. RT-PCR data also suggest the Clu 121 line expresses agouti-related protein (not shown). The gene expression profile observed is consistent with that of an Arc NPY neuron.

While differences did not reach significance, NPY mRNA expression shows a trend for increase at 2 h after OXA treatment (**Fig. 3**), consistent with in vivo data on OXA effects in the Arc. It is possible that we have not yet determined the optimal OXA dosing regimen or time course to observe significant changes in NPY.

Treatment with OXA resulted in a significant decrease in leptin receptor expression at 1 h post-treatment ($p = 0.014$; **Fig. 4**). Leptin receptor mRNA did not differ from controls at later timepoints.

Conclusions

Data suggest that in addition to increasing NPY expression and cell firing (as demonstrated by earlier in vivo and in vitro work, and consistent with trend for increased NPY here), OXA actions in the Arc might also result in changes in leptin sensitivity. Other reports suggest that LepR and orexin receptors may have convergent intracellular second messenger pathways, such as extracellular factor-regulated kinase and the Janus kinase JAK2/STAT3 pathway [11], which could interact to modulate activity of Arc NPY neurons. The effects of OXA on LepR mRNA in Clu 121 cells is intriguing. Leptin results in downregulation of PPAR-γ1 and increase in LepR in hepatic stellate cells, and increased PPAR-γ1 activation in these cells reduces LepR and block leptin effects [12]. Orexin is known to increase PPAR-γ2 in fat cells [13]. Although it is unknown whether orexin increases PPAR-γ1 in neuronal tissue, our preliminary data suggests a similar mechanism might be present in neuronal cells as evidenced by the OXA-induced changes in LepR observed here. Together our results suggest that in addition to orexin-leptin interactions in downstream signaling pathways, OXA actions in Arc might also result in short-term downregulation of LepR, thus temporarily decreasing the ability of leptin to shut down NPY production during OXA-stimulated feeding. We are currently working to futher investigate interactions between leptin and orexin signaling in the Clu 121 cell line. We further plan to use organotypic punches to validate our findings in intact isolated Arc tissue before moving to in vivo validation of our findings. Finally, as a long-term goal we hope to work towards the establishment of an Arc-specific adult cell line via immortalization of neurons extracted from microdissected Arc tissue.

Acknowledgements

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References

1. Yamanaka, A., et al., Orexin-induced food intake involves neuropeptide Y pathway. Brain Res, 2000. 859(2): p. 404-9.
2. van den Top, M., et al., Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus. Nat Neurosci, 2004. 7(5): p. 493-4.
3. Backberg, M., et al., Orexin receptor-1 (OX-R1) immunoreactivity in chemically identified neurons of the hypothalamus: focus on orexin targets involved in control of food and water intake. Eur J Neurosci, 2002. 15(2): p. 315-28.
4. Horvath, T.L., S. Diano, and A.N. van den Pol, Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. J Neurosci, 1999. 19(3): p. 1072-87.
5. Muroya, S., et al., Orexins (hypocretins) directly interact with neuropeptide Y, POMC and glucose-responsive neurons to regulate Ca 2+ signaling in a reciprocal manner to leptin: orexigenic neuronal pathways in the mediobasal hypothalamus. Eur J Neurosci, 2004. 19(6): p. 1524-34.
6. Lopez, M., et al., Neuropeptide Y, but not agouti-related peptide or melanin-concentrating hormone, is a target peptide for orexin-A feeding actions in the rat hypothalamus. Neuroendocrinology, 2002. 75(1): p. 34-44.
7. Baskin, D.G., et al., Leptin receptor long-form splice-variant protein expression in neuron cell bodies of the brain and co-localization with neuropeptide Y mRNA in the arcuate nucleus. J Histochem Cytochem, 1999. 47(3): p. 353-62.
8. Higuchi, H., A. Hasegawa, and T. Yamaguchi, Transcriptional regulation of neuronal genes and its effect on neural functions: transcriptional regulation of neuropeptide Y gene by leptin and its effect on feeding. J Pharmacol Sci, 2005. 98(3): p. 225-31.
9. Magni, P., et al., Control of the expression of human neuropeptide Y by leptin: in vitro studies. Peptides, 2001. 22(3): p. 415-20.
10. Belsham, D.D., et al., Generation of a phenotypic array of hypothalamic neuronal cell models to study complex neuroendocrine disorders. Endocrinology, 2004. 145(1): p. 393-400.
11. Funato, H., et al., Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. Cell Metab, 2009. 9(1): p. 64-76.
12. Lee, J.L., et al., A peroxisome-proliferator activated receptor-gamma ligand could regulate the expression of leptin receptor on human hepatic stellate cells. Histochem Cell Biol, 2007. 127(5): p. 495-502.
13. Digby, J.E., et al., Orexin receptor expression in human adipose tissue: effects of orexin-A and orexin-B. J Endocrinol, 2006. 191(1): p. 129-36.