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Orexin expression is regulated by alpha-melanocyte stimulating hormone

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Abstract

The hypothalamic melanocortin system plays a fundamental role in the regulation of energy homeostasis. Orexins (hypocretins) are also involved in a diverse range of physiological processes, including food intake. Previous evidence has suggested that hypothalamic orexin expression may be influenced by the central melanocortin system. Here, we studied orexin mRNA levels in *Pomc*^{-/-} mice, a mouse model lacking all endogenously produced melanocortin peptides. Orexin expression in the lateral hypothalamus was significantly increased in corticosterone deficient *Pomc*^{-/-} mice. Further, when circulating glucocorticoids were restored to levels within the physiological range, orexin expression remained elevated. However, ICV administration of the melanocortin α -MSH to *Pomc*^{-/-} mice reduced orexin expression back down to WT levels. This was independent of the effects of α -MSH on food intake as in *Pomc*^{-/-} mice pair-fed to α -MSH-treated animals elevated orexin expression persisted. These data indicate that α -MSH may play a role in the regulation of orexin expression in *Pomc*^{-/-}, with an elevation in orexin levels contributing to the hyperphagia seen in these animals.

Keywords

Food intake; glucocorticoids; hypothalamus; melanocortins; orexins; proopiomelanocortin

INTRODUCTION

Proopiomelanocortin (POMC) is a prohormone that undergoes extensive post-translation modification to generate a range of smaller, biologically active peptides. These include ACTH and α -, β -, and γ -melanocyte-stimulating hormone, collectively known as the melanocortins {Coll, Farooqi, et al. 2004 9216 /id}{Cone 2005 20600 /id}. POMC-expressing neurons within the arcuate nucleus (ARC) are critically involved in the integration of nutritional and hormonal signals and the regulation of energy homeostasis {Cone 2005 20600 /id}. These neurons project to second order neurons in hypothalamic regions known to regulate feeding behaviour, including the paraventricular nucleus (PVN),

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lateral hypothalamic area (LHA) and dorsomedial hypothalamic nucleus (DMH) {Cone 2005 20600 /id}. Extensive data from pharmacological and genetic studies have demonstrated that POMC-derived melanocortins suppress feeding through activation of the melanocortin 4-receptor (MC4-R) whereas the endogenous antagonist agouti-related protein (AgRP) stimulates food intake (for extensive review: {Coll, Farooqi, et al. 2004 9216 /id} {Cone 2005 20600 /id}).

In particular, MC4-R is highly expressed in the LHA {Kishi, Aschkenasi, et al. 2003 3981 /id} with anatomical evidence that POMC neurons send projections to neurons within the region expressing the orexigenic peptides, melanin-concentrating hormone (MCH) and orexin {Elias, Saper, et al. 1998 2296 /id}. We have previously studied the expression of MCH mRNA in the LHA of POMC-deficient mice (*Pomc*^{-/-}), a mouse model lacking all endogenously produced melanocortin peptides which develops hyperphagia and obesity. The elevation in MCH expression in these mice suggests that MCH neuronal activity is negatively regulated by melanocortin peptides and that elevated MCH signalling contributes to their hyperphagic behaviour {Challis, Coll, et al. 2004 8782 /id}.

Orexins (OX-A and OX-B, also called hypocretins) regulate food intake {López, Seoane, et al. 2002 2394 /id} through a mechanism which may involve NPY {Yamanaka, Kunii, et al. 2000 1469 /id} {Dube, Horvath, et al. 2000 1438 /id}. Orexin neurons are also responsive to peripheral signals regulating food intake, such as leptin {López, Seoane, et al. 2000 337 /id}, glucose {Cai, Widdowson, et al. 1999 2262 /id} and ghrelin {Toshinai, Date, et al. 2003 4714 /id}. A previous study investigating the link between the melanocortin system and orexin expression has shown that blockade of central melanocortin receptors does not affect orexin mRNA expression in the LHA {Hanada, Nakazato, et al. 2000 63 /id}. In this study, we have used *Pomc*^{-/-} mice to examine orexin expression in the total absence of endogenous melanocortin and have gone on to specifically investigate the effects of α -MSH on orexin mRNA levels in the LHA.

MATERIAL AND METHODS

Animals

We used adult (4-month old) male *Pomc*^{-/-} mice generated on a 129/SvEv background as previously described {Challis, Coll, et al. 2004 8782 /id} {Coll, Challis, et al. 2005 23785 /id}. Mice were maintained under controlled temperature (22 °C) and light (12 h light from 08:00–20:00 h) and had *ad libitum* access to water and standard chow. All experiments were conducted in accordance with United Kingdom Home Office Guidelines for the Care and Use of Laboratory Animals.

Glucocorticoid replacement in *Pomc*^{-/-} mice

Corticosterone (CORT) (Sigma-Aldrich, Poole, UK) replacement was given for 10 days as a drinking water supplement at a final concentration of 25 µg/ml. Control animals drank water (vehicle).

Intracerebroventricular α -MSH treatment

ICV cannulae were implanted as previously described {Tung, Piper, et al. 2006 33282 /id}. On d 0, mice underwent stereotaxic surgery to place an indwelling guide cannula into the lateral ventricle. Mice (wild-type and *Pomc*^{-/-} mice) were anaesthetised with a mix of inhaled isoflurane and oxygen and a 26-gauge steel guide cannula (internal diameter 0.24 mm, outer diameter 0.46 mm, length 2 mm; Semat International, Herts, UK) was implanted into the right lateral ventricle using the following coordinates: 1.0 mm lateral from bregma, 0.5 mm posterior to bregma. The guide cannula was secured to the skull using quick-drying

cyanoacrylate glue and a dental cement (Associated Dental Products, Wiltshire, UK) and a dummy cannula was inserted. All animals received analgesia (Rimadyl, 5 mg/kg; Pfizer Animal Health, Kent, UK) and antibiotic (Teramycin LA, 60 mg/kg; Pfizer Animal Health) before being returned to their home cage. On day 8, 1 h before the onset of the dark cycle, mice received either 2 nmol of α -MSH or PBS (vehicle) in a total volume of 2 μ l. This was administered using a Hamilton syringe over 2 min. Food intake was measured over the subsequent 24 h.

Sample collection

At the end of the experiments, animals were killed by cervical dislocation. Their brains were removed, frozen immediately on dry ice and then maintained at -80°C until processed. Trunk blood was collected in heparinized tubes, centrifuged and the plasma separated and kept frozen at -20°C until assayed. Animals from each experiment were sacrificed on the same day and at the same time (within a 1 hour interval). We used 7-11 animals/group.

Blood biochemistry analysis

Plasma corticosterone levels were measured using ELISA kits (Crystal Chem Inc; Downers Grove, IL, USA).

In situ hybridization

Coronal brain sections (16 μm) were cut on a cryostat, and immediately stored at -80°C until hybridization. A specific antisense oligo for orexin was used (5'-TTCGTAGAGACGGCAGGAACACGTCTTCTGGCGACA-3' (Mouse GenBank accession number: AF041242; 5' position: 240). In situ hybridizations were performed as previously published {López, Seoane, et al. 2002 2394 /id}{Nogueiras, Tovar, et al. 2004 11372 /id}{López, Lelliott, et al. 2006 27789 /id}. All sections were scanned and the specific hybridization signal was quantified by densitometry using a digital imaging system (ImageJ 1.33, NIH; Maryland, USA) {López, Seoane, et al. 2002 2394 /id}{Nogueiras, Tovar, et al. 2004 11372 /id}{López, Lelliott, et al. 2006 27789 /id}. We used 16-20 sections for each animal (4-5 slides with 4 sections per slide). The mean of these 16-20 values was used as the densitometry value for each animal. The coefficient of intra-assay variance was 5.4 % and the coefficient of inter-assay variance was 6.1 %.

Statistical analysis and data presentation

The data were expressed as mean $\pm$ SEM and analyzed using a computerized package for statistical analysis (StatView 4.57, Abacus Concepts; California, USA). Statistically significant difference was determined by ANOVA followed by a post-hoc multiple comparisons Bonferroni test. A $P < 0.05$ was considered as being significant. Orexin mRNA levels were presented as percentage change in relation to a relevant control (wild-type or vehicle-treated mice).

RESULTS

Pomc^{-/-} mice have increased orexin expression in the LHA which is not reversed with corticosterone replacement

Compared to WT littermates *Pomc*^{-/-} mice displayed a significant increase in the expression of orexin mRNA in the LHA (Fig. 1A and 1B). To determine if this upregulation of orexin expression was as a result of glucocorticoid deficiency, *Pomc*^{-/-} mice were given corticosterone supplemented drinking water for 10 days (CORT). Resulting circulating corticosterone levels were somewhat higher than those seen in previous studies {Coll, Challis, et al. 2005 23785 /id} but were within the physiological range (WT 224.4 ± 76.6 ng/

ml; *Pomc*^{-/-}: not detected; *Pomc*^{-/-} supplemented with CORT: 159.1 ± 74.1 ng/ml; P<0.001 WT vs. *Pomc*^{-/-} and *Pomc*^{-/-} vs. *Pomc*^{-/-} supplemented). CORT treated *Pomc*^{-/-} mice still had a significant increase in orexin expression (Fig. 1A and 1B).

Central administration of α-MSH to *Pomc*^{-/-} mice restores orexin expression to wild-type levels

To determine if increased orexin expression in *Pomc*^{-/-} mice was due to a lack of central melanocortin signalling, we administered a single dose of 2 nmol of α-MSH to *Pomc*^{-/-} mice {Tung, Piper, et al. 2006 33282 /id}. As expected, such treatment resulted in a reduction in food intake and body weight over the subsequent 24 hours (Fig. 2A and 2B). Analysis of orexin expression in these mice showed α-MSH treatment decreased orexin mRNA in *Pomc*^{-/-} mice to the level seen in wild-type mice ICV treated with vehicle (Fig. 3A and 3B). To determine if this effect was a consequence of the reduced food intake seen following α-MSH treatment, we measured orexin in *Pomc*^{-/-} mice pair-fed to the α-MSH-treated group. The elevation in orexin expression persisted in pair-fed *Pomc*^{-/-} mice (Fig. 3A and 3B) despite a reduction in weight that exactly matched the α-MSH-treated group (Fig. 2A).

DISCUSSION

Our results show that *Pomc*^{-/-} mice exhibit a marked increase in orexin mRNA levels in the LHA and that α-MSH may directly control the expression of orexin in this model.

There are anatomical data linking the melanocortin and orexin neuronal systems. POMC neurons in the arcuate nucleus project their axons towards orexin neurons in the LHA {Elias, Saper, et al. 1998 2296 /id} {Kishi, Aschkenasi, et al. 2003 3981 /id}, while these same arcuate neurons also express orexin receptors {Backberg, Hervieu, et al. 2002 2639 /id}. Functional *in vitro* data have also demonstrated that orexins may directly regulate POMC neurons in the ARC in a manner reciprocal to that of leptin {Muroya, Funahashi, et al. 2004 7468 /id}. There are also previous data investigating the link between the central melanocortin signalling and orexin expression. Hanada *et al* reported that pharmacological blockade of central melanocortin receptors with the synthetic melanocortin antagonist SHU9119 did not induce changes on orexin mRNA expression {Hanada, Nakazato, et al. 2000 63 /id}. Further, this group also studied neuropeptide expression in the LHA of *A^{y/a}* (*agouti*) mice. These mice ectopically overexpress the melanocortin antagonist *agouti* and although they had an increase in MCH expression, orexin expression remains unchanged from wild-type level. These authors concluded that interruption of signalling at MC4R activates MCH but not orexin gene expression.

Although we too have previously shown that MCH is upregulated in *Pomc* deficiency, another model of disrupted melanocortin signalling, the data presented herein on orexin expression in *Pomc*^{-/-} is in contrast to that of Hanada *et al*. The reasons for this discrepancy are not entirely clear but it may be that orexin and MCH neurons are differentially affected by prevailing melanocortin tone. Although Hanada *et al* studied mice with low melanocortin tone, achieved either by treatment with SHU9119 or as a result of *agouti* over-expression, in neither model was there a complete absence of POMC-derived peptides. This is in contrast to the *Pomc*^{-/-} mouse, which lacks *all* POMC-derived peptides, yet still expresses AgRP. Previous data have shown AgRP to preferentially activate orexin expression over MCH expression in the LHA {Zheng, Corkern, et al. 2002 2805 /id}. Furthermore, in the Hanada study, it is possible that any one of the many peptide products of POMC may have acted on LHA neurons, albeit at a low level, to maintain expression of orexin mRNA.

Glucocorticoids play a major role in body weight homeostasis, metabolism and food intake {Asensio, Muzzin, et al. 2004 14100 /id}. Our mouse model is also unusual among models of hypothalamic obesity in that it is also glucocorticoid deficient {Challis, Coll, et al. 2004 8782 /id} {Coll, Challis, et al. 2005 23785 /id}. Previous published data have demonstrated that circulating glucocorticoid levels may have an impact on hypothalamic orexin expression {Stricker-Krongrad & Beck 2002 2727 /id} {Drazen, Coolen, et al. 2004 7058 /id} {Ford, Al Barazani, et al. 2005 21999 /id}. For example, Drazen *et al.* reported that adrenalectomy significantly reduced hypothalamic orexin gene expression but that this could not be restored by glucocorticoids in the drinking water {Drazen, Coolen, et al. 2004 7058 /id}. Interestingly, in our study although the glucocorticoid deficient mice had an increase in orexin expression, we too found no change with addition of glucocorticoid supplemented drinking water, indicating that lack of glucocorticoids in the adult state is not the cause of the increased orexin expression observed in *Pomc*^{-/-} mice. Whether glucocorticoid lack during development has an impact upon orexin expression is not addressed in this study and merits further investigation. However, the data from the ICV and pair feeding experiments in this study suggest that the increased orexin expression is as a direct result of a loss of α -MSH activity.

The contribution of high orexin levels to the phenotype of *Pomc*^{-/-} mice is also potentially interesting. *Pomc*^{-/-} mice are markedly obese and have increased food intake {Yaswen, Diehl, et al. 1999 9875 /id} {Challis, Coll, et al. 2004 8782 /id} {Coll, Challis, et al. 2005 23785 /id}. We have previously demonstrated that this hyperphagic behaviour is associated with a specific increase in MCH expression in the LHA {Challis, Coll, et al. 2004 8782 /id} {Coll, Challis, et al. 2005 23785 /id}. Our current data suggest that an increase in the orexin expression in the LHA may also contribute to the hyperphagia observed in *Pomc*^{-/-} mice. To more robustly determine the significance of these observations, pharmacological manipulation of central orexin signalling may have been informative. For example, an amelioration of the hyperphagia seen in *Pomc* null mice following central administration of an orexin antagonist would lend further mechanistic support to the link between elevated orexin expression and the phenotype of *Pomc* null mice. However, given the arousal-promoting actions of orexins {Willie, Chemelli, et al. 2001 2550 /id}, an important caveat to such a study is that any reduction in food intake following pharmacological interruption of orexin neurotransmission may simply be a sequelae of reduced wakefulness. {Brisbare-Roch, Dingemans, et al. 2007 2721 /id}. Indeed, although the data in this study do not directly address the physiological relevance of the interaction between the melanocortin and orexin system, given the arousal-promoting actions of orexins {Willie, Chemelli, et al. 2001 2550 /id}, it is tempting to speculate that the anorectic actions of α -MSH may, in part, be related to a reduction in the activity and/or arousal state of the animals.

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ABBREVIATIONS

α-MSH	α -melanocyte stimulating hormone
ACTH	adrenocorticotrophic hormone
AgRP	agouti-related peptide
ARC	arcuate nucleus of the hypothalamus

CORT	corticosterone
ICV	intracerebroventricular
LHA	lateral hypothalamic area
MC4-R	melanocortin-4 receptor
MCH	melanin concentrating hormone
NPY	neuropeptide Y
OX	orexin
OX-A	orexin-A
OX-B	orexin-B
PF	pair-fed
POMC	proopiomelanocortin
<i>Pomc</i>^{-/-} mice	proopiomelanocortin knockout mice
WT	wild-type

FIGURE 1.

(A) Autoradiographic images of representative mice brain coronal sections, incubated with a ³⁵S labelled antisense orexin mRNA oligoprobe in the described groups. (B). Orexin mRNA levels in the LHA of the described experimental groups. ****: P<0.001 vs. wild-type.

FIGURE 2.

Changes in body weight (**A**), food intake (**B**) in WT mice ICV-treated with vehicle (Veh), *Pomc*^{-/-} mice ICV-treated with vehicle, α -MSH and pair-fed (PF, ICV-treated with vehicle). ***: P<0.001 vs. WT ICV-treated with vehicle; ###: P<0.001 between the labelled groups.

FIGURE 3.

(**A**) Autoradiographic images of representative mice brain coronal sections, incubated with a ³⁵S labelled antisense orexin mRNA oligoprobe in the described groups. (**B**) Orexin mRNA levels in the LHA of the described experimental groups. **: P<0.01 vs. WT ICV-treated with vehicle; #: P<0.05 and ##: P<0.01 between the labelled groups.