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CONTRIBUTION OF REGIONAL BRAIN MELANOCORTIN RECEPTOR SUBTYPES TO ELEVATED ACTIVITY ENERGY EXPENDITURE IN LEAN, ACTIVE RATS

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Abstract—Physical activity and non-exercise activity thermogenesis (NEAT) are crucial factors accounting for individual differences in body weight, interacting with genetic predisposition. In the brain, a number of neuroendocrine intermediates regulate food intake and energy expenditure (EE); this includes the brain melanocortin (MC) system, consisting of MC peptides as well as their receptors (MCR). MC3R and MC4R have emerged as critical modulators of EE and food intake. To determine how variance in MC signaling may underlie individual differences in physical activity levels, we examined behavioral response to MC receptor agonists and antagonists in rats that show high and low levels of physical activity and NEAT, that is, high- and low-capacity runners (HCRs, LCRs), developed by artificial selection for differential intrinsic aerobic running capacity. Focusing on the hypothalamus, we identified brain region-specific elevations in expression of MCR 3, 4, and also MC5R, in the highly active, lean HCR relative to the less active and obesity-prone LCR. Further, the differences in activity and associated EE as a result of MCR activation or suppression using specific agonists and antagonists were similarly region-specific and directly corresponded to the differential MCR expression patterns. The agonists and antagonists investigated here did not significantly impact food intake at the doses used, suggesting that the differential pattern of receptor expression may by more meaningful to physical activity than to other aspects of energy balance regulation. Thus, MCR-mediated physical activity may be a key neural mechanism in distinguishing the lean phenotype and a target for enhancing physical activity and NEAT.

Key words: non-exercise activity thermogenesis (NEAT), obesity, spontaneous physical activity, HCR/LCR.

INTRODUCTION

The problem of obesity continues to plague society, decreasing the quality of life and increasing mortality rates (Allison et al., 1999; Ogden et al., 2014). An obesogenic environment interacts with genetic predisposition to make an individual more or less susceptible to becoming obese. However, only a small proportion of human obesity can be accounted for by monogenic mutations; instead, genetic polymorphisms and their associated intermediates contribute to weight gain (Cauchi et al., 2009; Zegers et al., 2012). The brain leptin-melanocortin (MC) system is one of the most common systems to be a target of polymorphisms affecting energy homeostasis (Fan et al., 1997; Zemel and Shi, 2000; Butler, 2006; Lee et al., 2006). Of its five known receptors, MC receptors (MCRs) 3, 4, and 5 are present in the adult mammalian brain. MC4R is a common target for alterations related to both monogenic and polygenic obesity (Santini et al., 2009; Tao, 2010). Their roles in energy balance, particularly for MC4R and MC3R, have been demonstrated through studies using gene deletions as well as reports of single nucleotide polymorphisms (SNPs) in human obesity (Bell et al., 2005). MC4R mutations are among the most common monogenic causes of human obesity, with over 150 reported mutations (Tao, 2010), and genome-wide complex trait analysis indicating significant SNP signals surrounding MC4R as part of the ‘‘missing heritability” for BMI (Locke et al., 2015).

Daily physical activity influences both weight management and cardiovascular disease risk. The energy expenditure (EE) of daily living, called

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Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; DMN, dorsomedial nucleus; EE, energy expenditure; GAPDH, glyceraldehyde phosphate dehydrogenase; HCR, high-capacity runner; LCM, laser capture microdissection; LCR, low-capacity runner; MC, melanocortin; MCR, melanocortin receptor; MTII, melanotan II; NEAT, non-exercise activity thermogenesis; PeFLH, perifornical lateral hypothalamus; POMC, proopiomelanocortin; PVN, paraventricular nucleus; Q-PCR, quantitative PCR; RER, respiratory exchange ratio, VCO_2/VO_2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNPs, single nucleotide polymorphisms; TRH, thyrotropin releasing hormone; VMN, ventromedial nucleus.

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non-exercise activity thermogenesis (NEAT), contributes to inter-individual differences in body weight and resistance to fat gain during overfeeding (Levine et al., 1999). The tendency to be physically active is a biologically regulated, heritable trait [reviewed in Novak and Levine, 2007; Garland et al., 2011], but also interacts with known genetic determinants of obesity risk. Evidence links the brain MC system to physical activity as well, for example, in obese animal models, deletion of MCRs results in lower physical activity along with obesity (Chen et al., 2000a; Coll et al., 2004; Loos et al., 2005; Yako et al., 2012). Altogether, ample evidence implicates the brain MC system as a major contributing factor for individual differences in energy balance and physical activity.

In both rats and humans, high intrinsic aerobic capacity strongly associates with high levels of physical activity (Levine et al., 1999; Novak et al., 2009); both of these traits could serve to identify the lean phenotype, and could share common underlying mechanisms. In a rat model of leanness (Koch and Britton, 2001; Koch et al., 2012) that demonstrates consistently elevated levels of physical activity and NEAT (Novak et al., 2009, 2010; Shukla et al., 2012; Gavini et al., 2014; Smyers et al., 2015), we previously reported elevated levels of MCR expression in the hypothalamus (Shukla et al., 2012). The relevance of the divergent receptor profiles to behavior, including physical activity levels, is unexplained. Here, we determined the site specificity of receptor expression by using a highly specific methodology, laser capture microdissection (LCM). We then used MC receptor subtype-specific agonists and antagonists to examine the relevance of regional differences in MCR expression, including MC5R, to intrinsic differences in daily physical activity levels.

EXPERIMENTAL PROCEDURES

Rats

High-capacity runner (HCR) and low-capacity runner (LCR) female rats (generation 27–31) with overlapping ranges of body weight were provided by the International Aerobic Rat Resource maintained at the University of Michigan (Koch and Britton, 2001; Wisloff et al., 2005; Ren et al., 2013). Compared to males, HCR and LCR females show less change in body weight with age and less group difference in body weight, thus minimizing potential group bias stemming from differences in body weight and composition (Goran, 2005; White et al., 2009; Butler and Kozak, 2010; Tschop et al., 2012). Female rats were used throughout these studies unless noted otherwise. Although sex differences in metabolism and subtle variations in daily physical activity that are estrous-cycle related may exist (Smyers et al., 2015), the measured differences in activity between LCR and HCR rats are robust and not dependent on sex (Novak et al., 2010; Smyers et al., 2015), and variance related to estrous cycle has minimal effect on EE (Giles et al., 2010). Rats were housed individually and placed on a 12:12-h light:dark cycle with lights on at 0700 EST, and received *ad libitum* water and rodent chow (Lab Diet 5001; Lab Diet, Richmond, Indiana, USA). A total of 148 rats (HCR + LCR) were used in these studies; all procedures and handling were in accordance with and approved by Kent State University's Institutional Animal Care and Use Committee (IACUC).

LCM and gene expression

Brains from 12 HCR and 12 LCR rats were sectioned at 12-μm sections on a cryostat and mounted onto SuperfrostPlus slides. Sections were stained using a quick protocol to allow visual identification of the arcuate nucleus, perifornical lateral hypothalamus (PeFLH), paraventricular nucleus (PVN), ventromedial nucleus (VMN), and dorsomedial nucleus (DMN). We chose these regions because of their documented presence of MC receptors in these areas and the actions of MC on metabolism. Briefly, sections were fixed in a 75% EtOH (30 s), rinsed in water, and immersed in Hematoxylin (90 s), followed by serial dehydration (75%, 95%, and 100% EtOH for 30 s each) and immersion in xylenes (5 min). The LCM machine (Arcturus XT™) was used to identify and capture brain regions onto CapSure® HS LCM Caps (Molecular Devices), pooling 6–12 captures from one nucleus onto one cap for every sample. Pre- and post-capture images of the tissue confirmed accuracy of nuclei captured; we estimate that extra cells comprised less than 1% of the total captured material.

RNA from the LCM samples was isolated and measured using quantitative real-time PCR (Q-PCR). The samples were purified and total RNA was extracted using an RNA purification kit (Ribopure; Ambion Life Technologies, Grand Island, NY, USA). RNA concentration and purity were measured using NANODROP (ND-1000; Nanodrop Technologies, Wilmington, DE, USA) with A260/280 ratio ranging from 1.8 to 2.1; only samples with optimum RNA integrity numbers were used for further processing. Purified total RNA was reverse transcribed using the Applied Biosystems reverse transcription reagents kit (Carlsbad, CA, USA), using random hexamers with thermal cycling at 25 °C for 10 min, 48 °C for 30 min, 95 °C for 5 min. Next, 20–100 ng of cDNA was used for quantifying the expression of the genes of interest using Taqman probes (Applied Biosystems); starting concentration of cDNA was kept the same within the nuclei examined. All samples were run in triplicate on the StratageneMx3005P Real-Time PCR System (Agilent, Carlsbad, CA, USA), with annealing temperature of 60 °C, for 40 cycles. The housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) was used as control for all assays and the relative expression was calculated using the comparative Ct method (ΔCt) method (Schmittgen and Livak, 2008).

Brain micropunches and Western blot

Ten HCR and 10 LCR male rats were euthanized and brains were rapidly removed, frozen in cooled isopentane, and stored at −80 °C. Brains were sectioned at 100 μm on a cryostat; sections were placed onto slides and frozen on dry ice. Tissue sites

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