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DISCRETE MELANOCORTIN-SENSITIVE NEUROANATOMICAL PATHWAY LINKING THE VENTRAL PREMMAMILLARY NUCLEUS TO THE PARAVENTRICULAR HYPOTHALAMUS

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Abstract—The physiological effects of melanocortin-4 receptor (MC4-R) on metabolism have been hypothesized to be mediated individually or collectively by neuronal groups innervating the paraventricular nucleus of the hypothalamus (PVH). The present study was designed to identify MC4-R-expressing neurons that innervate the PVH using retrograde tract tracing techniques in the MC4-R-GFP reporter mice. Our initial mapping identified very limited projections from MC4-R-expressing neurons to the PVH. This included a defined population of MC4-R-positive neurons located in the ventral premmamillary nucleus (PMv). Anterograde tracing experiments confirmed projections from PMv neurons to the medial parvicellular subdivision of the PVH, in close proximity to oxytocin neurons and β-endorphin-containing fibers. Given the known stimulatory effects of leptin and sexual odorants exposure on many PMv neurons, it was expected that MC4-R-expressing neurons in the PMv might be responsive to leptin and activated by odors exposure. Contrary to expectation, MC4-R-GFP neurons in the PMv do not respond to leptin as demonstrated by double labeling for GFP and leptin-induced phosphorylated STAT3. However, we found that Fos expression is induced in a large subset of MC4-R-GFP neurons in the PMv in response to opposite sex odors. Collectively, these results provide evidence for a previous unrecognized role of MC4-R expressed by neurons innervating the PVH that are also sensitive to reproductive cues.

Key words: leptin, melanocortin, mouse, tracing, reproduction, oxytocin.

INTRODUCTION

The fundamental role of melanocortin-4 receptor (MC4-R) in body weight regulation is established given the morbid obesity observed in naturally occurring loss-of-function mutation of MC4-R in humans (Vaisse et al., 1998) and gene knock-out in mice (Huszar et al., 1997). MC4-R-expressing neurons regulate a variety of functions including food intake and energy expenditure (Cowley et al., 1999; Adage et al., 2001; Butler et al., 2001; Farooqi et al., 2003; Krakoff et al., 2008), lipid mobilization (Nogueiras et al., 2007), cardiovascular responses (Tallam et al., 2006; Greenfield et al., 2009; Skibicka and Grill, 2009), anxiety (Adan et al., 1999; Chaki et al., 2003) and several neuroendocrine axis (Fekete et al., 2000; Dhillo et al., 2002; Lu et al., 2003).

Given its varied effects, it is not surprising that the MC4-R is widely expressed in the central nervous system (Mountjoy et al., 1994; Kishi et al., 2003; Liu et al., 2003). To date, the critical neuronal groups through which MC4-R selectively exerts its effects still remain to be determined. It has been proposed that MC4-R-expressing neurons in the paraventricular nucleus of the hypothalamus (PVH) may play a key role in mediating the effects of MC4-R agonists. The PVH contains neurosecretory and hypophysiotropic neurons that innervate the pituitary gland and median eminence, and neurons that directly project to preganglionic autonomic nuclei in the brainstem and spinal cord (Swanson and Sawchenko, 1983). Thus, the PVH is a key site in the control of autonomic and endocrine functions, as well as ingestive behaviors. MC4-Rs are expressed in a subpopulation of PVH neurons that includes thyrotropin-releasing hormone, corticotropin-releasing hormone and oxytocin neurons (Mountjoy et al., 1994; Harris et al., 2001; Kishi et al., 2003; Liu et al., 2003; Lu et al., 2003). Substantial evidence suggests that the stimulation or blockade of MC4-R expressed by PVH neurons exclusively regulates feeding but not energy expenditure (Balthasar et al., 2005; Garza et al., 2008). Importantly, electrophysiological

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Abbreviations: BDA, biotin dextran amine; CTb, cholera toxin b subunit; DAB, diaminobenzidine tetrahydrochloride; GFP, green-fluorescent protein; LH, luteinizing hormone; MC4-R, melanocortin-4 receptor; PBS, phosphate-buffered saline; PMv, ventral premammillary nucleus; pSTAT3, phosphorylated-STAT3; PVH, paraventricular nucleus of the hypothalamus.

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data obtained in hypothalamic slices demonstrate that PVH neurons activity is regulated by presynaptic melanocortin receptors (MC4-R and/or MC3-R) located on axon terminals originating from local interneurons and/or neurons outside of the PVH (Cowley et al., 1999; Melnick et al., 2007). Therefore, injections of melanocortin receptors (MC4-R and/or MC3-R) ligands into the PVH which produce changes in feeding, energy expenditure and cardiovascular responses (Giraudo et al., 1998; Cowley et al., 1999; Skibicka and Grill, 2009), may possibly act at the presynaptic level. The former observations leave the possibility that MC4-R-expressing neurons afferent to the PVH might be important in mediating many of the physiological actions of MC4-R, and thus identifying these putative neurons might be critical to a better understanding of the functional organization of the central melanocortin system. Recently, our group characterized a mouse model in which the green-fluorescent protein (GFP) is expressed under the control of MC4-R promoter (Liu et al., 2003; Gautron et al., 2010), thus rendering the visualization of MC4-R-expressing neurons straightforward. Using this unique model of MC4-R-GFP mice, we sought to determine the brain sites which contain MC4-R-expressing neurons and innervate the PVH.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Adult males and females MC4-R-GFP and C57BL/6 (Jackson Laboratory) mice (8–16 weeks old) were maintained on a 12-h light/dark cycle and temperature-controlled environment, with free access to water and food. The MC4-R-GFP mice express Tau-Sapphire GFP under the control of the MC4-R promoter. The genetic background was an admixture of C57BL/6 and CBA. Our group has previously demonstrated that these animals faithfully express GFP in MC4-R-expressing neurons (Liu et al., 2003). Specifically, Liu and colleagues reported a complete agreement between GFP and MC4-R mRNA in a vast majority of brain sites including the PVH and ventral premammillary nucleus (PMv). GFP-positive neurons were found not to express MC4-R mRNA only in a few brain sites (the dentate gyrus, layer 1 of the cortex and medial cerebellar nucleus), most likely due to altered in situ hybridization signal. Furthermore, Ghamari-Langroudi and colleagues (Ghamari-Langroudi et al., 2011) recently demonstrated that all tested MC4-R-GFP neurons in the mouse PVH responded to MT-II and α-MSH. In the study of Liu and colleagues, only half of MC4-R-GFP neurons responded to MT-II. The latter observation does not necessarily imply that GFP is ectopically expressed but that MC4-R itself may be trafficked to the presynaptic terminals. One study showed MC4-R immunoreactivity in nerve terminals (Cowley et al., 1999). However, the antibody used in the latter study is not well-characterized and the presynaptic localization of MC4-R still remains a speculation. Mice were genotyped as described by Liu and colleagues (2003). All experiments were carried out in accordance with the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (1996) with approval of the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committees.

At the end of each procedure described below, all animals were deeply anesthetized with an intraperitoneal injection of chloral hydrate (500 mg/kg) and transcardially perfused with saline followed by 10% neutral buffered formalin. Brains were removed, post-fixed in 10% formalin for 2–4 h at room temperature, cryoprotected in 20% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 4 °C, and sectioned coronally at 25 μm into five series on a freezing microtome. Sections were stored in cryoprotectant solution (20% glycerol, 30% ethylene glycol in PBS) at −20 °C.

Tracer injections

Males and females MC4-R-GFP mice were deeply anesthetized with ketamine (5 mg/100 g) and xylazine (1 mg/100 g). The animals (n = 10 males, n = 4 females) received unilateral stereotaxic injections of the retrograde tracer cholera toxin b subunit (CTb 1%; List Biological Laboratories) into the PVH (−0.43 mm from bregma; +0.23 lateral; −4.75 mm from surface of the skull). The retrograde tracer was injected with a glass micropipette and air pressure injection system as described previously (Elmquist et al., 1998a; Elias et al., 1999). After 7 days, animals were perfused and their brains were dissected and processed as described above. We also injected the anterograde tracer biotin dextran amine (BDA) (10% in water, 10,000 MW; Invitrogen/Molecular Probes) into the PMv (−5.0 mm from the posterior end of the olfactory bulb; +0.4 mm lateral; −5.4 mm from dura mater) in C57BL/6 mice (n = 12 males and n = 10 females). Animals were perfused 10 days later and brains were processed as described above.

Leptin administration

A subgroup of male and female MC4-R-GFP (12–16 weeks) mice were fasted for 24 h and injected with recombinant murine leptin intraperitoneal (5 mg/kg, n = 3 males and n = 4 females; provided by A.F. Parlow, Harbor-UCLA Medical Center, Torrance, California, USA; through the National Hormone and Peptide Program) or pyrogen-free saline (n = 3 males and n = 3 females) (Sigma). All injections were given between 11 a.m. and 12 p.m. Animals were perfused 40 min later. Brains were dissected and sectioned as previously described. These animals were used to assess phosphorylated-STAT3 (pSTAT3) following leptin administration.

Histology

Retrograde tracing experiments. CTb-containing neurons and injection sites were stained using immunoperoxidase labeling. Brain sections were pre-treated with 0.3% hydrogen peroxide in PBS for 15 min at room temperature. Sections were incubated overnight in goat primary antiserum against CTb (1:50,000; List Biological Labs, Campbell, CA; cat#703) in 3% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) with 0.25% Triton X-100 in PBS (PBT), followed by biotinylated donkey anti-goat (Jackson Immunoresearch; cat#705065147), then incubated in a solution of avidin–biotin (1:1,000; Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) dissolved in PBS for 1 h. After washing in PBS, the sections were developed with diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.01% hydrogen peroxide (Sigma–Aldrich) resulting in a brown precipitate.

Colocalization of CTb and GFP was determined using double immunofluorescent labeling. Brain sections were incubated overnight at room temperature with primary antisera against GFP made in chicken (1:10,000; Aves Labs, Tigard Oregon; cat#GFP-1020) and against CTb made in goat (1:25,000) in 3% normal donkey serum PBT. Sections were washed and incubated with anti-chicken AlexaFluor-488-conjugated secondary antibody (1:1,000; Invitrogen/Molecular Probes;