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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 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.

Keywords

leptin; melanocortin; mouse; tracing; reproduction; oxytocin

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Conflict of interest: none.

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 regulate feeding but not energy expenditure (Balthasar et al., 2005, Garza et al., 2008). Importantly, electrophysiological 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 (Giraudou 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

1.1 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 12h 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 PMv. GFP-positive neurons were found not to express MC4-R mRNA only in a few brain sites (dentate gyrus, layer 1 of 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 response 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. On 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 μ m into 5 series on a freezing microtome. Sections were stored in cryoprotectant solution (20% glycerol, 30% ethylene glycol in PBS) at –20° C.

1.2 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 (Elmqvist 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.

1.3 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.

1.4 Histology

1.4.1 Retrograde tracing experiments—CTb-containing neurons and injection sites were stained using immunoperoxidase labeling. Brain sections were pretreated 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; cat#A11039) and anti-goat AlexaFluor-594-conjugated secondary antibody (1:1,000; Invitrogen/Molecular Probes; cat# A11058) for 1 h at room temperature. Another series of brain sections was labeled for GFP and β -endorphin. Sections were incubated in primary antisera against β -endorphin made in rabbit (1:2,000; Phoenix Pharmaceutical, Burlingame, CA; cat#H0-22-33) and anti-GFP made in chicken (1:10,000). Then, the tissue was washed and incubated in anti-chicken AlexaFluor-488-conjugated secondary antibody (1:1,000) and anti-rabbit AlexaFluor-594-conjugated secondary antibody (1:1,000; Invitrogen/Molecular Probes; cat# A21207) for 1 h at room temperature.

1.4.2. Anterograde tracer experiments—BDA that was injected into the PMv was detected using immunoperoxidase labeling. Briefly, brain sections were pre-treated with 0.3% hydrogen peroxide in PBS, followed by an overnight incubation in a solution of ABC in PBT without normal serum. After washing in PBS, the sections were incubated in a solution of 0.04% DAB and 0.01% hydrogen peroxide.

Another series of sections were submitted to triple labeling immunohistochemistry for BDA, β -endorphin and oxytocin. The tissue was incubated overnight in a solution of AlexaFluor-594-conjugated streptavidin (Invitrogen/Molecular Probes; cat#532356) in PBT without normal serum. On the next day, sections were incubated in a primary antiserum against β -endorphin made in rabbit (1:2,000; Phoenix Pharmaceutical), overnight at room temperature, followed by anti-rabbit AlexaFluor-350-conjugated secondary antibody (1:250; Invitrogen/Molecular Probes) for 1 h at room temperature. Subsequently, sections were incubated in oxytocin monoclonal antibody (1:2,500; Chemicon; MAB5296) overnight at room temperature. Sections were then incubated for 1 h in anti-mouse AlexaFluor-488-conjugated secondary antibody.

1.4.3. Colocalization of pSTAT3 and GFP—Colocalization for pSTAT3 and GFP was determined using double-label immunoperoxidase. Sections were pretreated with 1% NaOH and 1% hydrogen peroxide in distilled water for 15 min, followed by 0.3% glycine in PBS for 10 min, and 0.03% sodium dodecyl sulfate (SDS, Sigma-Aldrich) in PBS for 10 min. Then, sections were incubated overnight at room temperature in a rabbit polyclonal anti-pSTAT3 antibody (1:3,000; Cell Signaling, MA; cat#9131L), followed by a biotinylated donkey anti-rabbit secondary antibody, followed by a solution of ABC as described before. After washing in PBS, the sections were developed with DAB-nickel resulting in the accumulation of dark blue precipitate in the nucleus of leptin-responsive neurons. After several washes, the tissue was labeled for GFP using the previously mentioned chicken anti-GFP and DAB. It resulted in the brown cytoplasmic labeling of GFP-positive neurons.

1.4.4 Colocalization of Fos and GFP—For the experiments in which we performed odor behavioral test, series of sections were pre-treated with 0.3% hydrogen peroxide in PBS, followed by an overnight incubation in a primary antiserum against Fos made in rabbit (1:50,000; Oncogene, MA; cat#PC38). As described before, Fos protein was labeled using a biotinylated anti-rabbit antibody, ABC solution and DAB-nickel as chromogens. For double immunofluorescence, sections were incubated with primary antisera against Fos made in rabbit (1:30,000) and GFP made in chicken (1:10,000), followed by the corresponding AlexaFluor-conjugated secondary antibodies.

Fluorescently-labeled sections were mounted on gelatin-coated slides, air-dried and coverslipped with Vectashield mounting medium containing DAPI (Vector laboratories, Burlingame, CA; H-1500). DAB-labeled sections were mounted on gelatin-coated slides, air-dried, dehydrated in graded ethanols, cleared in xylenes, and coverslipped with Permaslip (Alban Scientific).

1.5 Odor tests

The PMv neurons are engaged in conspecific odors responses (Kollack-Walker and Newman, 1995, Yokosuka et al., 1999, Veening et al., 2005, Cavalcante et al., 2006, Donato et al., 2010). Thus, we designed two sets of experiments to assess whether MC4R-GFP neurons in the PMv respond to these stimuli. Experiments to assess PMv activation in response to odors exposure were performed with adult MC4R-GFP sexually naïve female mice (n= 6; control n= 3). On the day of experiment, females were transferred into a cage with soiled-bedding where adult male mice were kept. Animals of the control group were moved to a clean-bedding cage. Both groups were perfused following 2 h. Brains were dissected and processed as described previously.

1.6 Data analysis and production of photomicrographs

Brain sections were analyzed using a Zeiss microscope (Axioskop2) under epifluorescence and brightfield illumination. Digital images were captured using a digital camera (AxioCam) attached to the microscope and a desktop computer running the Axiovision 3.1 software. High resolution fluorescent images were generated using stacks of optical sections (between 5 to 8 sections covering a thickness of ~10µm) obtained with a Zeiss microscope (Imager ZI) attached to the Apotome system. Images were captured with a digital camera (AxioCam) attached to the microscope and a desktop computer running Axiovision 4.5. Drawings were generated using a camera lucida-equipped microscope, digitalized and incorporated in the software Adobe Illustrator CS2. Adobe Photoshop CS2 was used to combine the images into plates. Only minor adjustments to color balance, contrast and brightness were performed. Double labeled neurons were quantified using 10× or 20× objective under brightfield illumination (pSTAT3/GFP) or using epifluorescence microscopy (Fos or CTb/GFP). These data were not corrected for double counting or using a stereological technique because section thickness did not vary between groups. Moreover, we count only one representative section for each nucleus analyzed. Hence, our results are meant to provide relative data, but are not meant to be accurate estimates of absolute cell counts. Dual-labeled neurons were counted and percentages of the means ($\pm$ SEM) were estimated. The atlas level designations correspond to those described by Franklin and Paxinos (Paxinos and Franklin, 2001).

Results

1.1 Distribution of MC4R-GFP neurons projecting to the PVH

Predictably, different injection sites of CTb in the mouse PVH produced distinct results (Fig. 1A–G). Of 10 mice injected, 3 mice showed injections centered in the PVH (cases #143,146, 234) and 3 mice showed injections centered in the peri-PVH (cases #147,180, 233). In the

remaining animals, injections did not contain the PVH and therefore not considered for analysis. PVH-centered injections entirely filled the major subdivisions of the PVH, with some leakage outside of the PVH boundaries (Fig. 1A, B, C). Peri-PVH-centered injections filled only the dorsal, lateral and ventral hypothalamic areas immediately adjacent to the PVH (Fig. 1D, E, F). Case #147 also partially comprised the rostral subdivisions of the PVH (Fig. 1D). Injections centered in the peri-PVH were used as anatomical controls. Similar injections were performed in female mice with similar results (not shown).

Our results indicate that the distribution of retrogradely-labeled neurons in mice is similar to that previously reported in rats (Sawchenko and Swanson, 1983, Elmquist and Saper, 1996, Champagne et al., 1998, Pan et al., 1999, Fekete et al., 2004, Williamson and Viau, 2007). Briefly, CTb-labeled neurons were found in the limbic forebrain, the bed nucleus of the stria terminalis, multiple hypothalamic nuclei including the ventromedial and the dorsomedial nuclei, the arcuate nucleus, the medial preoptic area and the organum vasculosum of the lamina terminalis, as well as brainstem nuclei such as the periaqueductal gray, the parabrachial nuclei, the ventrolateral medulla and the nucleus of the solitary tract. Figure 2 illustrates the distribution of CTb-positive neurons in some of these structures (Fig. 2A–H). Thorough examination of the entire brain revealed that GFP- and CTb-positive neurons in the ipsilateral side of CTb injection formed two largely segregated populations. For example, few dual-labeled neurons (1 or 2 per section) were inconsistently seen in the bed nucleus of the stria terminalis (Fig. 2A), posterior hypothalamus (Fig. 2C), ventromedial nucleus of the hypothalamus (Fig. 2D), and the periaqueductal gray in its ventrolateral part only (Fig. 2F). Doubles were never observed in other structures containing both CTb and GFP including, but not limited to, the dorsomedial nucleus of the hypothalamus (Fig. 2B), nucleus of the solitary tract (Fig. 2E), A1 nucleus (Fig. 2G), or the parabrachial nucleus (Fig. 2H). In contrast, the ventral premammillary nucleus (PMv) contained dual-labeled neurons (Fig. 3A–D). In agreement with earlier work (Sawchenko and Swanson, 1983, Cullinan et al., 1996, Campeau and Watson, 2000), following the deposit of neuronal retrograde tracers in the PVH, numerous retrogradely-labeled neurons are found in the PMv (Fig. 3C). MC4-R-GFP neurons are found in the PMv (Fig. 3A, B), as reported by Liu and colleagues (2003), number of them showing significant colocalization with CTb (Fig. 3C, D). The percentage of CTb-positive neurons immunoreactive to GFP reached similar proportions in cases #143 (35%), #146 (25%), #234 (36%), #147 (26%).

1.2 Innervation of the PVH by PMv neurons

Anterograde tracing experiments were performed to confirm the existence of projections from PMv neurons to the PVH. The efferent projections from the PMv are well described in rats (Canteras et al., 1992, Rondini et al., 2004), however, we felt the necessity to re-evaluate these projections in mice since differences between species may occur. Moreover, the PMv is part of a sexually dimorphic brain circuit (Canteras et al., 1992). Thus, the results of our anterograde tracer injections were compared between males and females. We obtained six cases with BDA injections centered in the PMv (n=2 females: cases #268, #404, and n=4 males; #91, #277, #418, #419; Fig. 4A–G). The distribution of BDA-labeled fibers was very similar to what have been described for rats and no clear difference between males and females were noticed. Briefly, the PMv supplies a strong input to the anteroventral periventricular nucleus, to the periventricular nucleus of the hypothalamus and to the bed nucleus of the stria terminalis (Fig. 5A, B). We also found moderate to high innervation of the lateral septum, the medial septal nucleus, the horizontal diagonal band of Broca and the medial preoptic area. Moderate number of fibers also targeted the medial and the cortical nuclei of the amygdala, the retrochiasmatic area, the arcuate nucleus, the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus, the dorsal and ventral subdivisions of the dorsomedial nucleus of the hypothalamus and the periaqueductal

gray matter (Fig. 5C–H). Low innervation of the perifornical area and the lateral hypothalamic area, as well as of the posterior nucleus of hypothalamus and the supramammillary nucleus was observed. In agreement with previous studies performed in rats (Canteras et al., 1992), we also observed BDA-positive fibers in both the parvocellular and magnocellular subdivisions of the PVH (Fig. 6A–E). The density of fibers varied from case to case but we found that in all cases the medial parvocellular subdivision displayed a higher density of PVH varicose fibers and terminal-like structures (Fig. 6B, E). The PMv also innervated a region comprising the ventral peri-PVH and the anterior hypothalamic area (Fig. 6B). Our injections often resulted in undesired spread of tracer outside the boundaries of the targeted brain site. However, results obtained from differently positioned injection sites served as anatomical controls. Moreover, the full projection fields of anterogradely-labeled neurons and the distribution pattern of retrogradely-labeled neurons were very comparable across injected animals and similar to that reported in the literature.

We evaluated the potential innervation of PMv neurons by melanocortin projections by also assessing the distribution of β -endorphin immunoreactive terminals (Fig. 6C, E). Virtually all pro-opiomelanocortin neurons in the mouse arcuate nucleus also contain β -endorphin (Cowley et al., 2001). Our findings show the absence of β -endorphin fibers in the PMv (not shown) which suggests that MC4-R action in PMv neurons mostly occurs at the presynaptic level, i.e. within the PVH. In agreement with such a view, β -endorphin immunoreactive fibers are abundant in the PVH, in its medial parvocellular subdivision (Fig. 6A). These fibers are rarely in close apposition to MC4-R-GFP neurons which rather aggregated in the posterior PVH and the medial magnocellular subdivision (Fig. 6A, B). Our triple labeling experiments further confirmed that BDA- and β -endorphin-positive fibers converge to the medial parvocellular PVH (Fig. 6B–D), a region which also contained many oxytocin neurons (Fig. 6A). At higher magnification, oxytocin neurons were innervated by both BDA- and β -endorphin-positive terminal-like contacts (Fig. 6E). These observations are in agreement with the hypothesis that MC4-R signaling in the PVH may possibly occur on presynaptic terminals originating from the PMv.

1.3 Responsiveness of PMv MC4-R-expressing neurons to leptin versus conspecific interactions

Leptin receptors are robustly expressed in the PMv (Elmqvist et al., 1998b, Elias et al., 2000, Leshan et al., 2009, Scott et al., 2009). Here, we investigated whether MC4-R is expressed in leptin-sensitive neurons using pSTAT3 as a marker for direct leptin signaling. As reported before (Munzberg et al., 2003), the peripheral administration of leptin induces a dense expression of pSTAT3 immunoreactivity in different regions of the hypothalamus and brainstem, including the preoptica area, the retrochiasmatic area, the arcuate nucleus, the lateral hypothalamic area, the dorsomedial and ventromedial nuclei of the hypothalamus, the PMv and the dorsal vagal complex. Because many of these areas also express MC4-R (Mountjoy et al., 1994, Liu et al., 2003), we determined whether neurons which express MC4-R are responsive to leptin. Our results show some degree of GFP and pSTAT3 colocalization in the anterior levels of the lateral hypothalamic area (around 50%), in the posterior nucleus of the hypothalamus (40%), in the nucleus of the solitary tract and in the dorsal motor nucleus of the vagus nerve (both around 15%) (Table 1). Of note, we found virtually no colocalization of pSTAT3 and GFP immunoreactivity in the PMv of male or female mice (Fig. 7A, B). No colocalization between GFP and pSTAT3 was ever found in other areas analyzed including the medial preoptic area, the periventricular nucleus, the PVH, the retrochiasmatic area, the arcuate nucleus, dorsal and ventral subdivisions of the dorsomedial nucleus of the hypothalamus, the dorsal raphe and the parabrachial nucleus (not shown).

Previous studies have demonstrated that the PMv is activated during odors-triggered behaviors (Kollack-Walker and Newman, 1995, Yokosuka et al., 1999, Veening et al., 2005, Cavalcante et al., 2006, Donato et al., 2010). Using Fos protein as a marker of neuronal activation, we observed robust activation of PMv neurons following exposure to soiled bedding (Fig. 8A–C). Strikingly, we observed a relative high expression of Fos in MC4-R-GFP neurons ($33 \pm 3\%$ neurons; $n=6$) in females exposed to soiled bedding. We also found that $10 \pm 1\%$ of Fos-positive neurons colocalized with GFP. These results suggest the specific involvement of a subset of MC4-R-expressing neurons of the PMv in responding to reproductive relevant cues such as sexual odors.

Discussion

In the present study, we identified the ventral premammillary nucleus (PMv) as the exclusive brain site in which MC4-R-expressing neurons innervate the PVH. In addition, we showed that MC4-R neurons in the PMv are activated by opposite sex odors exposure, but do not respond to leptin administration.

Given the well-documented effects of melanocortin agonists on a wide range of autonomic and neuroendocrine functions, MC4-R signaling on PVH neurons has been the focus of several studies. Thus far, electrophysiological studies have demonstrated that melanocortin receptors signaling occurs at both the postsynaptic and presynaptic level within the rat PVH (Cowley et al., 1999, Melnick et al., 2007, Ghamari-Langroudi et al., 2011), suggesting a complex mechanism of actions of MC4-R within the PVH. While one study showed MC4-R immunoreactivity in nerve terminals within the PVH (Cowley et al., 1999), unfortunately, the cellular localization of MC4-R in the PVH will remain unclear until a more reliable MC4-R antiserum becomes available. Several studies reported a robust induction of *c-fos* in the PVH of rodents administered with melanotan-II or α -MSH (Caquineau et al., 2006, Kublaoui et al., 2006, Glavas et al., 2007), which suggested a stimulatory effect on PVH neurons, in agreement with the well-documented catabolic effects of MC4-R. Moreover, it is known that MC4-R-expressing PVH neurons regulate feeding (Balthasar et al., 2005) and exclusively project to the brainstem (not the median eminence) (Ghamari-Langroudi et al., 2011).

MC4-R-expressing nerve terminals within the PVH might also contribute to regulate PVH functions. According to our anatomical observations, these nerve terminals principally originate from the PMv. Notably, the vast majority of PMv neurons is glutamatergic (Ziegler et al., 2002, Donato et al., 2010), which suggests that MC4-R in PMv neurons might contribute to the stimulatory effects of melanocortin agonists on PVH neurons. Among the PVH neurons targeted by the PMv nerve terminals are oxytocin neurons. Interestingly, one study revealed multiple mechanisms of action of α -MSH on oxytocin neurons; while α -MSH inhibits the firing of oxytocin neurons, it also increases the dendritic release of oxytocin via a calcium-dependent pathway (Sabatier et al., 2003). Collectively, our findings suggest that MC4-R signaling in afferents to the PVH is limited to a subset of PMv neurons which project to the vicinity of β -endorphin terminals and oxytocin neurons in the medial parvocellular PVH. The PMv is anatomically part of a sexually dimorphic vomeronasal brain circuitry and is connected to areas related to reproductive control (Canteras et al., 1992, Rondini et al., 2004, Leshan et al., 2009). Notably, PMv neurons are stimulated by copulation (Kollack-Walker and Newman, 1995, Coolen et al., 1996, Veening et al., 2005) and exposure to opposite sex odors (Kollack-Walker and Newman, 1995, Yokosuka et al., 1999, Veening et al., 2005, Cavalcante et al., 2006, Leshan et al., 2009, Donato et al., 2010). Additionally, bilateral lesions of the PMv prevent the rise in luteinizing hormone (LH) secretion in response to odor stimulation (Beltramino and Taleisnik, 1985) and blunt the reproductive systems of female rats, causing a transient anestrus and a subsequent condition

of decreased estrogen and LH secretion across the estrous cycle (Donato et al., 2009). These data demonstrate the key role of the PMv in the various aspects of reproductive functions. However, PMv lesioned animals displayed no changes in body weight and food intake, suggesting that the deficits generated by the absence of PMv neurons are circumscribed to the reproductive physiology. The PMv also contains a dense collection of leptin-responsive neurons. We have recently shown that lesions of the PMv preclude the ability of leptin to restore fasting-induced suppression of LH secretion (Donato et al., 2009) and that re-expression of leptin receptors in the PMv of leptin receptor null mice induce puberty and improve fertility of otherwise infertile females (Donato et al., 2011). Interestingly, the MC4-R-expressing neurons in the PMv did not display pSTAT3 following leptin administration, thus forming a subset of PMv neurons which are distinct from those responsive to leptin. The latter observation suggests that MC4-R and leptin receptor signaling in the PMv are segregated and, therefore, may subserve different functions.

Although MC4-R expression in the PMv had been previously reported in mice (Liu et al., 2003), we are not aware of any prior anatomical or functional data regarding MC4-R-expressing neurons in the PMv. Our findings suggest that MC4-R-expressing neurons are specifically stimulated by odorants from the opposite sex. The latter results indicate that MC4-R-expressing neurons in the PMv may play a role in modulating relevant reproductive cues conveyed to the PVH. Of note, melanocortin agonists exert various actions on sexual function in mice and men. MC4-R agonists increase lordosis and solicitation behavior in female rats (Pfaus et al., 2004, Rossler et al., 2006), exert a well-documented pro-erectile action in rodents and men (Wessells et al., 2000, Molinoff et al., 2003, Giuliano et al., 2006, Allard et al., 2008) and stimulate the activity of GnRH neurons in mice (Israel et al., 2012). Conversely, melanocortin antagonists delays sexual behaviors in rats (Caquineau et al., 2006). Hence melanocortins agonists are considered as good candidates currently under clinical trials to treat sexual dysfunctions (Wikberg and Mutulis, 2008). Our study implies that the PMv may be one site of action of melanocortin agonists on sexual behaviors in mice. Furthermore, oxytocin neurons may be implicated in the mechanisms whereby MC4-R-expressing neurons in the PMv regulate reproductive functions. Although electron microscopy is the technique of choice for establishing synaptic connections between neurons, our own findings indicate that oxytocin neurons are in close vicinity to both melanocortin innervations and terminals from PMv neurons. Interestingly, Fos protein is induced in oxytocin neurons of animals exposed to odors of the opposite sex (Nishitani et al., 2004), and oxytocin released in the brain plays an important role in facilitating social and sexual behaviors in mice (Insel et al., 1997, Winslow and Insel, 2002). As mentioned earlier, α -MSH is known to mediate increased dendritic release of oxytocin (Sabatier et al., 2003), and indirect neuroanatomical and pharmacological evidence suggest that the release of dendritic oxytocin would contribute to the pro-sexual activity of melanocortin agonists (Caquineau et al., 2006). Projections from the PMv to other brain sites such as the medial amygdala and the preoptic area should also be considered. In this regard, it would be interesting to complete additional tracing studies to disclose the projection pattern of MC4-R-expressing neurons of the PMv. In summary, based on our anatomical findings, we propose that MC4-R signaling in PMv afferents to the PVH modulate the endocrine, behavior and autonomic response to relevant reproductive cues such as odorant stimuli.

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Highlights

- MC4-R-expressing neurons located in the PMv project to the mouse PVH.
- Melanocortin and PMv innervations converge onto oxytocin neurons.
- PMv MC4-R-expressing neurons are stimulated by sex odorants.

Figure 1.

Injections of the retrograde tracer cholera toxin-b (CTb) into the paraventricular nucleus of the hypothalamus (PVH). Camera lucida line drawings of three rostral-to-caudal levels of the PVH illustrating the placement of CTb injection sites in 3 cases in which the deposit was centered in the PVH itself (A–C) and 3 cases in which the deposit was centered in the peri-PVH (D–F). Outlined areas depict the extent of individual injection site with corresponding animal code numbers. DAB-labeled sections (brightfield illumination) illustrate CTb injection site in case 234 (G). Abbreviations: 3v, third ventricle; f, fornix; ox, optic chiasm; PaAP, paraventricular anterior parvicellular nucleus; PaPo, posterior paraventricular nucleus; PaM, paraventricular medial magnocellular nucleus; PaDC, dorsal cap of the

paraventricular nucleus; PaMP, medial parvicellular part of the paraventricular nucleus; PaV, parvicellular ventral part of paraventricular nucleus. Scale bar in G is 100 μ m.

Figure 2.

Distribution of GFP immunoreactivity (MC4-R-GFP) and neurons which project to the paraventricular nucleus of the hypothalamus (PVH). Dual-label immunohistochemistry for CTb (AlexaFluor-594) and GFP (AlexaFluor-488) shows absence or little colocalization of GFP- and CTb-positive neurons in the mouse brain (Apotome reconstruction). CTb accumulated in the cytoplasm of retrogradely-labeled neurons in structures ipsilateral to the side of injection such as the bed nucleus of the stria terminalis (A), the posterior hypothalamus (C), the ventromedial nucleus of the hypothalamus (D). Colocalization of CTb and GFP was seen only in few neurons in these structures and not consistently seen across animals. Doubles were never found in other brain structures such as the dorsomedial

nucleus of the hypothalamus (B), the nucleus of the solitary tract (E), the parabrachial nucleus (F), the A1 (G). White arrows indicate colocalization (yellow). Abbreviations: 3v, third ventricle; A1, noradrenergic group A1; Arc, arcuate nucleus of the hypothalamus; BST, bed nucleus of the stria terminalis; DMD, dorsal part of the dorsomedial nucleus of the hypothalamus; vDMH, ventral part of the dorsomedial nucleus of the hypothalamus; cDMH, compact part of the dorsomedial nucleus of the hypothalamus; DMV, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; PAGvl, ventrolateral periaqueductal grey; PBld, dorsolateral part of the parabrachial nucleus; PBle, external lateral part of the parabrachial nucleus; PBlv, ventrolateral part of the parabrachial nucleus; PH, posterior hypothalamus; ts, tract of the solitary; VMH, ventromedial nucleus of the hypothalamus. Scale bar = 50 μ m.

Figure 3.

A subset of MC4-R-GFP neurons of the ventral premammillary nucleus (PMv) project to the PVH. Dual-label immunohistochemistry for CTb (AlexaFluor-594) and GFP (AlexaFluor-488) showing colocalization in the PMv (epifluorescence and Apotome reconstruction). Abundant GFP immunoreactivity is seen in the mouse PMv (A, B: detail of boxed area). CTb accumulated in the cytoplasm of retrogradely-labeled neurons forming typical clusters encompassing part of the cell body (C). Colocalization of CTb and GFP was seen in many neurons (D). White arrows indicate double-labeled neurons. Abbreviations: 3v, third ventricle; f, fornix; PMv, ventral premammillary nucleus. Scale bar in A = 100 μ m; scale bar in B–D = 20 μ m.

Figure 4.

Injections of the anterograde tracer biotin dextran amine (BDA) into the ventral premammillary nucleus (PMv). Camera lucida line drawings represent the injection sites of BDA in each injected mouse (B–G). Animal code numbers are indicated. Each dot represents one cell that incorporated BDA. Sometimes BDA accumulated in a densely stained core in which individual cells are not distinguishable (shaded areas). Fluorescence photomicrograph showing one representative injection site in case #277 (A). Abbreviations: 3v, third ventricle; f, fornix; mt, mammillothalamic tract.

Figure 5.

Anatomical distribution of BDA-positive projections (AlexaFluor-594) originating from the ventral premammillary nucleus (PMv) in the brain of case #277 (A–H). Abbreviations: 3v, third ventricle; ac, anterior commissure; AHA, anterior hypothalamic area; Aq, aqueduct; Arc, arcuate nucleus of the hypothalamus; AVPV, anteroventral periventricular nucleus; BST, bed nucleus of the stria terminalis; DMH, dorsomedial nucleus of the hypothalamus; f, formix; HDB, horizontal limb of the diagonal band of Broca; LS, lateral septum; lv, lateral ventricle; MPO, medial preoptic area; MEApd, posterodorsal part of the medial amygdala; MEApv, posteroventral part of the medial amygdala; ox, optic chiasm; opt, optical tract; PAG, periaqueductal grey; PH, posterior hypothalamus; PVT, paraventricular thalamus;

RCA, retrochiasmatic area; VMH, ventromedial nucleus of the hypothalamus; Scale bar: A–H = 400 μm .

Figure 6.

Varicose fibers and synaptic buttons originated from the ventral premammillary nucleus (PMv) are in close apposition to oxytocin neurons and β-endorphin fibers in the medial parvicellular subdivision of the paraventricular nucleus of the hypothalamus (PVH). Triple-label immunohistochemistry for oxytocin (oxy, AlexaFluor-488), biotin dextran amine (BDA, AlexaFluor-594) and β-endorphin (β-end, AlexaFluor-350) immunoreactivity showing the distribution of melanocortin neurons and PMv neurons projections relative to the position of oxy-immunoreactive neurons in the PVH of case #277 (A–D). Within the medial parvicellular subdivision, many β-end-immunoreactive fibers and BDA fibers were seen seemingly contacting oxytocin neurons (epifluorescence and Apotome reconstruction)

(E). Boxed areas highlight visible terminal-like endings positive for β -end (short arrows) and BDA (thin arrows). Abbreviations: 3v, third ventricle; PaDC; dorsal cap of the paraventricular nucleus; PaM, paraventricular medial magnocellular nucleus; PaMP, medial parvicellular part of the paraventricular nucleus. Scale bar: A–D = 100 μ m; E = 10 μ m.

Fig 7. MC4-R-GFP neurons in the ventral premammillary nucleus (PMv) do not respond to leptin. Dual-label immunohistochemistry for pSTAT3 (black nuclei) and GFP (brown cell bodies) immunoreactivity showing that MC4-R-expressing neurons and leptin-responsive neurons form two distinct populations in the PMv (A,B). Brightfield photomicrographs are taken from the PMv of a female mouse (B: enlarged view of A). Black arrows show two representative GFP-positive neurons. Abbreviations: f, fornix. Scale bar: A = 100 μ m, B = 40 μ m.

Figure 8.

MC4-R-GFP neurons in the ventral premammillary nucleus (PMv) are responsive to opposite sex odor stimulation. Brightfield photomicrographs showing the distribution of Fos (black nuclei) immunoreactivity in the PMv of a naïve female mouse exposed to clean bedding (A) or a female mouse exposed to soiled bedding (B). Dual-label immunohistochemistry for Fos (AlexaFluor-594) and GFP (AlexaFluor-488) immunoreactivity in the PMv of a female mouse exposed to soiled bedding (C). Note that Fos is induced in numerous GFP-positive neurons in the animal exposed to soiled bedding (white arrows). Abbreviations: 3v, third ventricle; Arc, arcuate nucleus of the hypothalamus;

f, fornix; PMv, ventral premammillary nucleus. Scale bar in A and B = 200 μm ; scale bar in C = 50 μm .

Table 1

Double-labeled (GFP and pSTAT3 immunoreactive) neurons in selected brain nuclei.

region	Atlas level	Total GFP	doubles	% doubles/total GFP
LHA1	42	19.75 ± 2.6	10 ± 2.1	51.1 ± 11.9
LHA2	48	27.75 ± 2.9	1.75 ± 2.21	6.3 ± 8.3
PH	52	25.5 ± 3.8	11 ± 2.82	42.6 ± 4.45
NTS	94	40 ± 3.65	6.25 ± 1.5	15.75 ± 4.1
DMV	96	13 ± 1.8	2.25 ± 1.25	16.7 ± 7.3

Values represent estimates of mean counts of cells ± SEM (n=4). LHA1: rostral level of the lateral hypothalamic area, LHA2: caudal level of the lateral hypothalamic area, PH: posterior hypothalamus, NTS: nucleus of the solitary tract, DMV: dorsal motor nucleus of vagus nerve. The atlas level designations correspond to those described by Paxinos and Franklin (2001).