Experimental Neurology 232 (2011) 176–184

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Regular Article

Altered position of cell bodies and fibers in the ventromedial region in SF-1 knockout mice

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ARTICLE INFO

Article history:
Received 11 May 2011
Revised 14 July 2011
Accepted 22 August 2011
Available online 30 August 2011

Keywords:
Steroidogenic factor 1
Ventromedial hypothalamus
Cellular organization
Immunocytochemistry

ABSTRACT

The ventromedial nucleus of the hypothalamus (VMH) is a key cell group in the medial-basal hypothalamus that participates in the regulation of energy balance. Previous studies have shown that the cellular organization of the VMH is altered in mice with a disruption of the steroidogenic factor-1 (*NR5a1*) gene (SF-1 KO mice). The present study examined orexigenic/anorexigenic peptides (neuropeptide Y (NPY), agouti-related peptide (AgRP) and cocaine- and amphetamine-regulated transcript (CART)) and neural connections to and from the VMH in SF1 KO mice. NeuroVue tracing and Golgi staining were used to evaluate connections between the preoptic area (POA) and VMH and the orientation of dendrites in the VMH, respectively. Results of this study reveal changes in the cytoarchitecture of the region of the VMH with respect to the distribution of immunoreactive NPY, AgRP and CART. In WT mice projections from the POA normally surround the VMH while in SF-1 KO mice, projections from the POA stream through the region that would otherwise be VMH. Golgi impregnation of the region revealed fewer dendrites with ventrolateral orientations and in general, more variable dendritic orientations in SF-1 KO mice providing additional evidence that the connectivity of cells in the region is likely altered due to the cellular rearrangements consequent to disruption of the *NR5a1* gene. In conclusion, this study greatly extends the data showing that the morphology of the regions containing the VMH is disrupted in SF-1 KO mice and suggests that changes in the location of cells or fibers containing NPY, AgRP and CART may, in part, account for changes in body weight homeostasis in these mice.

Introduction

The ventromedial nucleus of the hypothalamus (VMH or VMN) is a bilateral cell group in the medial basal hypothalamus. Neurons in the VMH are involved in control of energy homeostasis (Brobeck, 1946; Iwamoto et al., 1999; Majdic et al., 2002; Powley, 1977), sexual behavior (Flanagan-Cato et al., 2006; Matsumoto and Yamanouchi, 2000; Yahr and Greene, 1992), parental behavior (Harding and McGinnis, 2005), anxiety (Zhao et al., 2008) and defensive behavior (Staples et al., 2005), and other physiological processes (Hosoi et al., 1999; Iwamoto et al., 1999). Older studies suggested that the VMH acts as a satiety center as VMH lesions in rats caused hyperphagia and obesity (Brobeck, 1946). While this hypothesis lost ground as other more selective lesion studies indicated the involvement of other nearby cell groups (arcuate and paraventricular nuclei), it has returned with more recent studies (King, 2006), including genetic mouse models that suggest roles for the VMH in the regulation of energy balance (Butler et al., 2000; Majdic et al., 2002). The role of the VMH in the control of multiple processes and behaviors is complex due to its close interconnection with other sites, including the amygdala and the preoptic area (POA) (Choi and Dallman, 1999; Saper et al., 1976; Ter Horst and Luiten, 1987).

Steroidogenic factor 1 (NR5A1, SF-1) is a major regulator of expression of steroidogenic enzymes and other genes in steroidogenic and non steroidogenic tissues (reviewed in Parker and Schimmer, 1997). Outside the central nervous system, SF-1 is expressed in adrenal glands, gonads, pituitary, placenta and spleen (Ikeda et al., 1993; Ingraham et al., 1994; Katoh-Fukui et al., 2005). In the central nervous system, SF-1 transcripts were detected in the diencephalon of mice on E11.5 (Ikeda et al., 1994) 5–6 days before the VMH becomes distinguishable based on Nissl stains. As the nucleus develops, SF-1 is selectively expressed in dorsomedial (VMHdm) and central parts (VMHc) of the VMH. Disruption of the *NR5a1* gene results in alterations of the cytoarchitecture of the VMH in SF-1 KO mice (Budefeld et al., 2008; Davis et al., 2004; Dellovade et al., 2000; Ikeda et al., 1995; McClellan et al., 2006) that also lead to changes in the terminal differentiation of the remaining VMH neurons (Tran et al., 2003). Changed cytoarchitecture of the VMH in SF-1 KO mice is likely dependent in part on alterations in the migration of neurons from the adjoining ventricular proliferative zone of the third ventricle (Dellovade et al., 2000, 2001; Tobet, 2002).

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doi:10.1016/j.expneurol.2011.08.021

Several aspects of VMH morphology are sexually dimorphic and gonadal steroid hormones are thought to be a major factor contributing to the development of these differences (Dorner and Staudt, 1969; Matsumoto and Arai, 1986; Micevych et al., 1987; Mong et al., 1999). Sex differences in the number of calbindin D-28 k immunoreactive (ir) cells were found in the VMH region in adult mice lacking SF-1 that are not exposed to endogenous gonadal steroid hormones, suggesting a genetic influence on the development of this sex difference (Budefeld et al., 2008).

SF-1 KO mice exhibit complete adrenal and gonadal agenesis (Ikeda et al., 1993) and develop obesity in adult life when adrenal transplants are used to keep them alive. Obesity is not a consequence of dysregulation of corticosteroid secretion since corticosterone levels in adult SF-1 KO mice are normal (Majdic et al., 2002). The present study was conducted to investigate the distributions of cells and fibers that were immunoreactive for the metabolic peptides agouti-related peptide (AgRP), neuropeptide Y (NPY) and cocaine- and amphetamine-regulated transcript (CART) in the region of the VMH in brains of adult WT and SF-1 KO mice. These peptides were chosen for their importance as metabolic peptides in the hypothalamus (Dietrich and Horvath, 2009). A fourth one, melanocortin or α-MSH, was not included in this study because it was examined in SF-1 KO mice previously (G.M.; unpublished data) and did not reveal obvious differences in the distribution of α-MSH. The aim of this study was to more fully characterize the alterations of the VMH region when *NR5a1* is genetically disrupted with special emphasis on the distribution of orexigenic/anorexigenic peptides that might partially explain the obese phenotype observed in these mice. Furthermore, immunoreactive arginine vasopressin (AVP) fibers that normally surround the VMH, connections between the preoptic area and the VMH using carbocyanine dye tracing, and a Golgi impregnation of the dendritic organization of cells in the region of the VMH were all analyzed to further characterize potential alterations in the connectivity of the VMH region in adult SF-1 KO mice. Differences in the immunoreactive locations for all of these peptides in the VMH region between WT and SF-1 KO mice suggest that the lack of SF-1 function plays a major role in the organization of orexigenic/anorexigenic pathways in the VMH region.

Material and methods

Animals and tissue recovery

C57BL/6J SF-1 heterozygous mice (SF-1+/− backcrossed to C57BL6/J for more than 10 generations and inbred for more than 10 generations) were bred to generate SF-1 KO and littermate wild type (WT) control mice. Mice were maintained on a 12:12 dark–light cycle with phytoestrogen free chow (Global 16% protein rodent diet (2016), Harlan Teklad, Bicester, Oxfordshire, UK) and water *ad libitum*. Normally, SF-1 KO mice die within 24 h of birth due to adrenal insufficiency. Therefore, all newborn pups (WT and SF-1 KO) received daily subcutaneous (s.c.) injections of 50 μl of corticosteroids in corn oil (400 μg/ml hydrocortisone (Sigma, Steinheim, Germany), 40 ng/ml dexamethasone (Sigma) and 25 ng/ml fludrocortisone acetate (Sigma) until animals were genotyped on days 6–7 postnatally using a PCR assay. Adrenal glands were transplanted into SF-1 KO pups from WT female donors on postnatal days 7 or 8 as described previously (Majdic et al., 2002). After adrenal transplantation, pups received four more s.c. injections of corticosteroids until weaning at 21 days. Control WT male and female mice (+/+ genotype) were derived from the same litters as SF-1 KO mice or in rare cases (when no littermates were available) from other age-matched litters, received corticosteroid injections postnatally, and were gonadectomized after weaning before the onset of puberty between P21 and P25. Body weights were measured once weekly from three weeks until sacrifice. For gonadectomies, WT mice were anesthetized with a mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW). On the day of sacrifice at 6 months of age, mice were anesthetized with a mixture of ketamine, xylazine and acepromazine and perfused with 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer (PB, pH = 7,3). After dissection from the skull, brains were post-fixed in the same fixative overnight at 4 °C and then stored until immunocytochemical processing in 0.1 M PB at 4 °C. All animal experiments were done according to ethical principles and in accordance with EU directive (86/609/EEC). Animal experiments were approved by the Veterinary commission of Slovenia and the Animal Care and Use Committee at Colorado State University.

SF-1 genotyping and sex determination

Tissue from mice 6–7 days of age were used to determine SF-1 genotype and chromosomal sex. DNA samples were obtained by tail clipping and digested in a thermostatic shaker in 200 μl of PCR DNA buffer (Promega, Madison, WI, USA) containing 0.15 mg of Proteinase K (Sigma) at 55 °C overnight. 3 μl of lysate was used for PCR reaction to determine the presence of WT or KO SF-1 allele and the presence or absence of Sry gene as described previously (Luo et al., 1994).

Immunocytochemistry on floating sections

Brains were embedded in 5% agarose (Sigma) and sectioned at 50 μm in cold 0.05 M PBS using a vibrating microtome (Integraslice 7550 MM, Campden Instruments, UK). Sections were incubated in 0.1 M glycine (Sigma) in 0.05 M PBS for 30 min followed by incubation in 0.5% sodium borohydride (Sigma) for 15 min at 4 °C. Glycine and sodium borohydride were washed out with 15 min and 20 min washes in 0.05 M PBS. Sections were blocked in 5% normal goat serum (Chemicon, Temecula, CA, USA) containing 0.5% Triton X-100 (Sigma) and 1% H_2O_2 (Merck, Darmstadt, Germany) for 30 min at 4 °C. Rabbit primary antibodies against arginine vasopressin (AVP, 1:15,000, ImmunoStar, Hudson, WI, USA), AgRP (1:4000, Phoenix Pharmaceuticals, Belmont, CA, USA), CART (1:10,000, Phoenix Pharmaceuticals) and NPY (1:10,000, Dia Sorin, Stillwater, MN, USA) were diluted in 0.05 M PBS containing 1% bovine serum albumin (Sigma) and 0.5% Triton X-100. Sections were incubated with primary antibodies over 3 nights at 4 °C with shaking. Sections were then washed in 0.05 M PBS containing 1% normal goat serum and 0.02% Triton X-100 four times 15 min at room temperature. Biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) against primary rabbit antibodies were diluted 1:500 in 0.05 M PBS containing 1% normal goat serum and 0.5% Triton X-100. Sections were incubated with secondary antibodies for 2 h, followed by 4 washes (15 min each) in 0.05 M PBS buffer containing 0.02% Triton X-100. Streptavidin–HRP complex (Jackson Immunoresearch) was diluted 1:2000 in 0.05 M PBS solution containing 0.5% TritonX-100. Sections were incubated with Streptavidin–HRP for 1 hour at room temperature and then washed in Tris-buffered saline (0.05 M Tris–HCl/0.9% NaCl; pH 7.5; Sigma) for 1 hour at room temperature. Antigen–antibody complexes were visualized as a black reaction product by incubating sections in 0.025% 3′3′-Diaminobenzidine/ammonium nickel (II) sulfate substrate (Sigma) in Tris-buffered saline (pH 7.5) containing 0.02% H_2O_2 for 5 min at room temperature. After mounting, sections were dried and coverslipped using hydrophobic medium (Pertex, Burgdorf, Germany). Immunocytochemical controls included omission of the primary antiserum and validation of immunoreactivity with patterns of distribution from prior publications.

Brain labeling

Micro-strips of lipophilic carbocyanine dye NeuroVue (PTI Research, Exton, PA, USA) were inserted into POA of intact brains (5 WT and 5 SF-1 KO mice) from the direction of the base of the brain.