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Fezf1 is a novel regulator of female sex behavior in mice

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ABSTRACT

Female sexual behavior is a complex process regulated by multiple brain circuits and influenced by sex steroid hormones acting in the brain. Several regions in the hypothalamus have been implicated in the regulation of female sexual behavior although a complete circuitry involved in female sexual behavior is not understood. Fez family zinc finger 1 (Fezf1) gene is a brain specific gene that has been mostly studied in the context of olfactory development, although in a recent study, *FEZF1* has been identified as one of the genes responsible for the development of Kallman syndrome. In the present study, we utilized shRNA approach to downregulate *Fezf1* in the ventromedial nucleus of the hypothalamus (VMN) with the aim to explore the role of this gene. Adult female mice were stereotaxically injected with lentiviral vectors encoding shRNA against *Fezf1* gene. Mice injected with shRNA against Fezf1 had significantly reduced female sexual behavior, presumably due to the downregulation of estrogen receptor alpha (ERα), as the number of ERα-immunoreactive cells in the VMN of Fezf1 mice was significantly lower in comparison to controls. However, no effect on body weight or physical activity was observed in mice with downregulated Fezf1, suggesting that the role of Fezf1 in the VMN is limited to the regulation of sexual behavior.

Significance statement: Fezf1 gene has been identified in the present study as a regulator of female sexual behavior in mice. Regulation of the female sexual behavior could be through the regulation of estrogen receptor alpha expression in the ventromedial nucleus of the hypothalamus, as the expression of this receptor was reduced in mice with downregulated Fezf1. As expression of Fezf1 is very specific in the brain, this gene could present a potential target for the development of novel drugs regulating hypoactive sexual desire disorder in women, if similar function of FEZF1 will be confirmed in humans.

1. Introduction

Female sexual behavior is a complex process regulated by numerous brain circuits and strongly influenced by sex steroid hormones secreted by the gonads (Nelson, 2005b). Female sexual behavior is thought to be mostly regulated by sex steroid hormones, secreted from the ovaries and acting at different brain circuits. Two main regions thought to be especially important for the regulation of female sexual behavior are the preoptic area and the ventromedial nucleus of the hypothalamus (VMN). Many cells in these two regions express estrogen receptor alpha (Erα) (Lauber et al., 1991) which is needed for the expression of female sexual behavior (Ogawa et al., 1998; Pfaff and Sakuma, 1979).

The mediobasal hypothalamus, part of which is the VMN, consists of several distinct brain nuclei and has many functions in the regulation of autonomic processes. Although some histologically distinct nuclei in the mediobasal hypothalamus seem to have single, well-defined roles in the regulation of autonomic processes, the VMN has more than one function. Previous studies have shown that the VMN is involved in the regulation of energy balance, defensive behavior and female sexual behavior (Colpaert, 1975; King, 2006; Majdic et al., 2002; Pfaff and Sakuma, 1979). These behaviors seem to be regulated by different parts of the VMN, with cells important for energy balance and defensive behavior being located primarily in the dorsomedial part of the VMN and cells involved in the regulation of female sex behavior in the ventrolateral part of the VMN. Because of that, the VMN is particularly interesting nucleus, and it is still not well understood how different roles of the VMN are regulated and executed. This is at least partially due to a small number of specific genes that are specifically expressed in the VMN, or even subsets of VMN neurons such as the dorsomedial or ventrolateral parts of the nucleus. A study by Kurrasch et al. (Kurrasch et al., 2007) identified only six genes that are specifically expressed in the VMN. These genes were transcription factors SF-1, Nkx2.1, Idb2, Fezf1, COUP-*TFII* and Satb2, of which only the roles of SF-1 and nkx2.1 were clearly defined, with SF-1 having a specific role in the hypothalamic development and function (Ikeda et al., 1995; Majdic et al., 2002), and nkx2.1 being important for the development of the whole

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hypothalamus (Marin et al., 2002). Another four genes were implicated in neural development before, but their specific functions in the hypothalamus have not been defined (Britanova et al., 2005; Caqueret et al., 2006; Tripodi et al., 2004). Therefore, we were interested to examine the role of Fezf1 in the VMN due to its specific expression pattern in the mediobasal hypothalamus.

Fez family zinc finger 1 (*Fezf1*) gene is a member of the Fez family of transcription factors currently composed of two members, *Fezf1* and *Fezf2*, and they were both identified through their specific expression in the anterior neuroepithelium of Xenopus and zebrafish embryos (Eckler and Chen, 2014). In mammals, *Fezf1* is expressed in the olfactory epithelium, but also very specifically in several regions of the hypothalamus including the preoptic region, VMN and medial amygdala (Eckler and Chen, 2014; Kurrasch et al., 2007). Both *Fezf1* and *Fezf2* have been shown to be important for the development of the olfactory system in mammals (Eckler et al., 2011; Watanabe et al., 2009). One recent study suggested that *Fezf1* gene is one of the genes responsible for the development of Kallmann syndrome (Kotan et al., 2014), a common cause of infertility due to deficient migration of GnRH producing neurons from the olfactory placode (Fechner et al., 2008). This is interesting, as *Fezf1* is expressed in the olfactory placode (Eckler and Chen, 2014) and in the hypothalamus (this study), and therefore, FezF1 might be an important link between these two brain regions. However, the connection and especially the role of *Fezf1* in this connection remains unclear.

In the present study, we therefore explored the role of functional Fezf1 expression in the VMN in connection with the known function of this nucleus, regulation of female sex behavior, body weight regulation and physical activity levels.

2. Material and methods

2.1. Animals and brain recovery

C57BL/6 J WT mice were originally obtained from Harlan (Italy) and bred at the University of Ljubljana, Veterinary Faculty, in standard conditions with 12–12 h light/dark cycle (lights on at 5 am and off at 5 pm) and food (phytoestrogen free diet; Harlan Teklad Diet 2016, Harlan, Milan, Italy) and water ad libitum. Mice were weaned at 21 days of age. In the first cohort of mice, 70- to 80-day-old gonadally intact male (WTM; $n = 3$) and gonadally intact female mice (WTF; $n = 12$) in different stages of the estrous cycle were euthanized in adulthood and used for immunohistochemical analyses. Stages of the estrous cycle were determined based on the unstained native vaginal smear cytology.

A functional analysis of Fezf1 in the VMN using a lentiviral-based gene knockdown approach in vivo was performed on a separate cohort of mice. Gonadectomized WT female mice were injected with non-targeting shRNA (WT-control, $n = 6$) or anti-Fezf1 shRNA ($n = 7$). Lentiviral particles were injected into the VMN bilaterally, and after two-week recovery, mice were tested for physical activity and female sexual behavior. Four days after the last sex behavior test (around P125), mice were anesthetized with the mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW), and perfused with transcardial fixation with 4% paraformaldehyde. Dissected brains remained in the same fixative over night at 4 °C and then stored in 0.1 M PB at 4 °C until further processing for immunohistochemistry.

All animal experiments were approved by the Veterinary Administration of the Republic of Slovenia and were done according to ethical principles, EU directive (2010/63/EU), and NIH guidelines.

2.2. Ovariectomy and hormone replacement

All female mice used in behavioral analyses were ovariectomized bilaterally at 60 days of age (after puberty) to eliminate endogenous gonadal steroids. Mice were anesthetized as described above and ovaries were excised through a single incision. Mice received two injections of butorfanol (Turbogesic, Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) after ovariectomy to alleviate potential pain.

To regulate circulating estradiol levels during the testing of female sexual behavior, mice received subcutaneous implants containing 17β-estradiol 3-benzoate (EB; Sigma, Steinheim, Germany). Silastic implants (1.02 mm inner diameter, 2.16 mm outer diameter) were filled 5 mm in length with crystalline EB diluted 1:1 with cholesterol (Sigma) (Wersinger et al., 1999) and closed on both ends by medical silastic adhesive (Dow Corning, MI, USA). Implants were inserted subcutaneously in the midscapular region under general anesthesia as described above. These implants yield plasma estradiol levels close to the physiological range normally observed during estrus (Wersinger et al., 1999). Sexual behavior tests were performed at least 10 days after implantation to allow the mice to fully recover from the surgery. Approximately 4 to 8 h before each behavioral test, females were injected subcutaneously with 0.8 mg of progesterone (P; Sigma) dissolved in 50 μl of corn oil (Sigma).

2.3. Stereotaxic injections

Lentiviral particles containing shRNA against the exon 4 of the 3'UTR of the mouse *Fezf1* gene (SH-055602-03) under the control of the constitutive hCMV promoter were purchased from Thermo Scientific (DE Healthcare Dharmacon, Lafayette, CO). Female adult mice 60 to 70 days old were anesthetized as described above and their heads placed into a motorized stereotaxic apparatus (Stoelting, Dublin, Ireland). A Hamilton syringe with a 33-gauge blunt tip needle (1701, Bonaduz, Switzerland) was stereotaxically placed into the VMN (1.3 mm posterior to bregma, 0.25 mm laterally to midline, 5.65 mm ventral to cortical surface area) using Neurostar stereo-drive software. Mice received bilateral injections of 2 μl of lentiviral particles (10^8 TU/ml) in a delivery medium at the rate of 0.4 μl/min. After the infusion, the cannula was lifted 50 μm, left in place for 5 min and then progressively retracted at the rate of 2 mm/min. Non-targeting control lentiviral particles (S-005000-01) with the same titer were used as negative controls.

2.4. Body weight and physical activity

Body weight was measured with a laboratory balance weekly for 5 weeks, starting 1 week after stereotaxic injections. Physical activity was recorded using the MotorMonitor frame cage rack system (Kinder Scientific, Poway, CA). Mice were housed individually in testing cages (21 cm × 36 cm floor area) with fresh bedding and water and food ad libitum 3 days prior the activity test to allow habituation to the novel environment that is usually associated with elevated physical activity (T.B. and G.M. unpublished observations). Physical activity was monitored for 7 consecutive days and quantified using the total distance travelled and time spent moving around per each testing day.

2.5. Female sexual behavior

All mice were tested for sexual behavior after completed physical activity monitoring. Female sexual behavior tests were performed in clear glass aquaria (17 cm high with 41.5 × 26 cm floor area) with a mirror positioned under the testing arena to obtain better views of facets of sexual behaviors (Wersinger et al., 1997). Females were tested during the first 2 to 4 h of the dark period of the circadian cycle, under dim red light illumination, and the test sessions were videotaped for