Neuroscience Letters 558 (2014) 109–114

Contents lists available at ScienceDirect

Neuroscience Letters

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

Androgen regulates development of the sexually dimorphic gastrin-releasing peptide neuron system in the lumbar spinal cord: Evidence from a mouse line lacking *androgen receptor* in the nervous system

Hirotaka Sakamoto[a,*], Kazuhiro Saito[a], Clarisse Marie-Luce[b,c,d], Kalina Raskin[b,c,d], Takumi Oti[a], Keita Satoh[a], Kei Tamura[a], Tatsuya Sakamoto[a], Sakina Mhaouty-Kodja[b,c,d]

[a] *Ushimado Marine Institute (UMI), Graduate School of Natural Science and Technology, Okayama University, Ushimado, Setouchi, Okayama 701-4303, Japan*
[b] *Centre National de la Recherche Scientifique (CNRS), Unité Mixte de Recherche (UMR) 7224, 9 quai St Bernard, 75005 Paris Cedex 05, France*
[c] *Institut National de la Santé et de la Recherche Médicale (INSERM) U952, 9 quai St Bernard, 75005 Paris Cedex 05, France*
[d] *Université Pierre et Marie Curie, 75005 Paris Cedex 05, France*

HIGHLIGHTS

- Androgen regulates masculine features primarily through androgen receptors (ARs).
- The gastrin-releasing peptide (GRP) system also mediates male sexual behavior.
- Testosterone may differentiate and maintain the activation of the spinal GRP system.
- We show that *AR* deletion attenuates GRP neurons and potentially sexual behavior in males.

ARTICLE INFO

Article history:
Received 10 October 2013
Received in revised form 27 October 2013
Accepted 30 October 2013

Keywords:
Androgen receptor
Sexual behavior
Testosterone
Gastrin-releasing peptide
Spinal cord
Mutant mouse

ABSTRACT

Androgens including testosterone, organize the nervous system as well as masculine external and internal genitalia during the perinatal period. Androgen organization involves promotion of masculine body features, usually by acting through androgen receptors (ARs). We have recently demonstrated that the gastrin-releasing peptide (GRP) system in the lumbar spinal cord also mediates spinal centers promoting penile reflexes during male sexual behavior in rats. Testosterone may induce sexual differentiation of this spinal GRP system during development and maintain its activation in adulthood. In the present study, we examined the role of ARs in the nervous system regulating the development of the sexually dimorphic GRP system. For this purpose, we used a conditional mouse line selectively lacking the *AR* gene in the nervous system. *AR* floxed males carrying (mutants) or not (controls) the nestin-Cre transgene were castrated in adulthood and supplemented with physiological amounts of testosterone. Loss of *AR* expression in the nervous system resulted in a significant decrease in the number of GRP neurons compared to control littermates. Consequently, the intensity of GRP axonal projections onto the lower lumbar and upper sacral spinal cord was greater in control males than in mutant males. These results suggest that ARs expressed in the nervous system play a significant role in the development of the GRP system in the male lumbar spinal cord. The *AR*-deletion mutation may attenuate sexual behavior and activity of mutant males *via* spinal GRP system-mediated neural mechanisms.

1. Introduction

Early in life, androgens such as testosterone (T) induce external and internal genitalia to develop into a masculine form and also masculinize the developing nervous system. This results in a permanent organization of neural populations and synaptic connections underlying male sexual behavior [8,10,13]. In adulthood, T acts to activate these neural areas including brain regions (*e.g.*, medial amygdala, bed nucleus of stria terminalis, and medial

* Corresponding author at: Ushimado Marine Institute (UMI), Graduate School of Natural Science and Technology, Okayama University, 130-17 Kashino, Ushimado, Setouchi, Okayama 701-4303, Japan. Tel.: +81 869 34 5210; fax: +81 869 34 5211.
E-mail address: hsakamo@uml.okayama-u.ac.jp (H. Sakamoto).

0304-3940/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2013.10.068

preoptic area) and spinal nuclei. In the spinal cord, androgen organization and activation of neural populations usually occurs by acting through androgen receptors (ARs) [8,10]. However, whether these processes involve the peripheral or central AR remain to be precised. We have recently demonstrated that the gastrin-releasing peptide (GRP) system in the lumbar spinal cord also mediates spinal centers promoting penile reflexes during male sexual behavior in rats [17,19]. We have also reported androgenic effects on the GRP system in the lumbar spinal cord in two animal models, one involving castration and T replacement in adult male rats and the second using genetically XY male rats carrying the testicular feminization (Tfm) allele of the AR gene [20]. Both animal models indicate that androgen signaling plays a pivotal role in the development of the spinal GRP system and in the regulation of GRP expression in the male lumbar spinal cord. However, in these animal models, it is difficult to distinguish between central and peripheral effects of androgens, including behavioral modulations. In the present study, we therefore used a conditional mouse line selectively lacking the *AR* gene in the nervous system [14] to elucidate whether central AR is important for the sex differentiation of the spinal GRP system, which plays a crucial role in male sexual behavior [19]. Using this mouse model in the study of the spinal GRP system is of great interest because this conditional mutation in mice interferes with the expression of male sexual behavior [14].

2. Materials and methods

2.1. Animals and genotyping

Control males with a floxed AR allele (AR^{fl}/Y) and their mutant littermates (AR^{fl}/Y, Nes-Cre; AR^{NesCre}) expressing Cre recombinase under the control of the promoter and the nervous system enhancer of nestin (*Nes*) gene were obtained as previously described [14]. The AR^{NesCre} mouse line was then backcrossed for at least nine generations into strain C57BL6J. Control and mutant males obtained in the same litters were weaned at 24–26 days of age and group housed under a controlled photoperiod (12 h light, 12 h dark cycle; lights on at 07:00 h) and temperature (22 °C) and given free access to food and water. The Cre transgene and floxed or excised *AR* alleles were identified by PCR analysis as previously described [4,14]. All studies were performed in accordance with the guidelines for care and use of laboratory animals [National Institutes of Health (NIH) Guide] and French and European legal requirements (Decree 87-848, 86/609/ECC). The experimental procedures have also been authorized by the Committee for Animal Research, Okayama University, Japan.

2.2. Gonadectomy and T supplementation

Adult males (8–10 weeks of age) of control (AR^{fl}/Y) and AR^{NesCre} were castrated under general anesthesia (xylazine/ketamine). At the time of castration, all males received 1 cm subcutaneous SILASTIC implants (3.18 mm outer diameter × 1.98 mm inner diameter; Dow Corning, Midland, MI, USA), either empty or filled with 10 mg T (Sigma–Aldrich, St.-Quentin Fallavier, France) and sealed at each end with SILASTIC adhesive. Animals were killed 3–4 weeks later. Circulating levels of T under these conditions were previously published elsewhere [7,14,15]. Circulating T was in the highest physiological range and similar comparable between controls and mutants treated with T.

2.3. Immunohistochemistry and immunofluorescence

Mice were anesthetized and transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). Lumbar spinal cords were quickly removed and immersed in the same fixative for 4 h at room temperature. After immersion in 25% sucrose in 0.1 M PB at 4 C for cryoprotection until they sank, the preparations were quickly frozen using powdered dry ice and cut into 30 μm-thick cross or horizontal sections on a cryostat (CM3050 S, Leica, Nussloch, Germany). We performed immunohistochemical analysis according to our established methods [18–20]. In brief, endogenous peroxidase activity was eliminated from the sections by incubation in a 1% H_2O_2 absolute methanol solution for 30 min followed by three 5-min rinses with phosphate buffered saline (PBS) (pH 7.4). These processes were omitted for the immunofluorescence method. After blocking nonspecific binding components with 1% normal goat serum and 1% BSA in PBS containing 0.3% Triton X-100 for 1 h at room temperature, sections were incubated with primary rabbit antiserum against GRP (1:5000) (Phoenix Pharmaceuticals, Burlingame, CA, USA) for 48 h at 4 °C. Immunoreactive (ir) products were detected with a streptavidin-biotin kit (Nichirei, Tokyo, Japan), followed by diaminobenzidine development according to our previous method [19]. The specificity of the antiserum was published previously [19]. GRP-ir cells in the spinal cord were localized using an Olympus Optical (Tokyo, Japan) BH-2 microscope. To determine the effect of central ARs on the projection site of GRP-ir axons, double-immunofluorescence staining of GRP and neuronal nitric oxide synthase (nNOS) (A-11; mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:5000 dilution), a marker protein for neurons in the sacral parasympathetic nucleus (SPN), was performed as described previously [19]. Alexa Fluor 546-linked anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-linked anti-rabbit IgG, both raised in goats (Molecular Probes), were used at a 1:1000 dilution for detection. Immunostained sections were imaged with a confocal laser scanning microscopy (Fluoview 1000, Olympus, Tokyo, Japan). For quantitative analysis, GRP-ir cells with clearly visible round nuclear profiles were counted in the anterior part of the lumbar spinal cord (L3–L4 level). To determine the optical density (OD) of positive GRP-ir fibers in the SPN, dorsal gray commissure (DGC) and dorsal horn (DH), at least six sections per animal were analyzed using ImageJ software (ImageJ 1.36b) with a set threshold level. The GRP-ir fiber pixel density was quantified as the average pixel density in three regions of each animal (SPN, DGC and DH), and were calculated as the ratio to the density seen in the DH in control males. At least five animals were used for these analyses in each group. The numbers and optical density of the GRP-ir neurons in the lumbar spinal cord were expressed as the mean ± standard error of the mean (S.E.M.) in each group, and were analyzed by a Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

Using immunohistochemical staining for GRP, we first examined the number of GRP-ir neurons located in the lumbar spinal cord (L3–L4 level; containing somata and dendrites of the GRP neurons) of control and AR^{NesCre} male mice (Fig. 1). The numbers of GRP-positive somata in the lumbar spinal cord of mice were 277.3 ± 14.1 and 134.0 ± 10.4 in the control and AR^{NesCre} males, respectively (Fig. 1). The number of GRP-ir neurons was significantly fewer in AR^{NesCre} males than in control males in the upper lumbar spinal cord ($n = 5$, $t = 8.24$, $P < 0.05$) (Fig. 1). Because all the mice in this study were castrated and received long-term (3–4 weeks) T supplementation, levels of circulating T were maintained at a high physiological range, comparable to that between controls and mutants as previously reported [7,14,15]. Then, we examined the projection of GRP-ir fibers to the SPN in the lower spinal cord (L5–L6 and S1 level; containing GRP neuronal axon terminals) of male mice because SPN provides autonomic preganglionic