Theriogenology 102 (2017) 183–189

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

SB223412, a neurokinin-3 receptor-selective antagonist, suppresses testosterone secretion in male guinea pigs

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ARTICLE INFO

Article history:
Received 14 February 2017
Received in revised form
28 July 2017
Accepted 29 July 2017
Available online 31 July 2017

Keywords:
NK3R
KNDy neurons
Kisspeptin
Dynorphin A
Contraceptive

ABSTRACT

Guinea pigs are important zoo animals and have been recommended for animal-assisted activities or therapy, however there are problems concerning testosterone inducing aggressive or sexual behaviors in male guinea pigs. Testicular testosterone secretion is regulated by pulsatile gonadotropin releasing hormone (GnRH)/luteinizing hormone (LH) release in mammals. The mechanism generating GnRH/LH pulses is thought to be governed by kisspeptin neurons, which coexpress neurokinin B (NKB) and dynorphin A (Dyn), in the arcuate nucleus (ARC). Kisspeptin neurons in the ARC are frequently referred to as KNDy neurons. The purpose of this study was to examine whether the antagonization of NKB-neurokinin-3 receptor (NK3R) signaling can manipulate testosterone secretion in male guinea pigs. A single subcutaneous administration or 7 days of oral administration of an NK3R-selective antagonist, SB223412 (50 mg/body), significantly decreased plasma testosterone levels in male guinea pigs. *In vitro* binding assays confirmed that SB223412 has a high affinity to guinea pig NK3R. These results suggest that SB223412 could be used as an orally-available compound to suppress testosterone levels in male guinea pigs. Double labeling *in situ* hybridization of kisspeptin and either NKB or Dyn showed that kisspeptin-expressing neurons contained NKB (77.9%) or Dyn (62.3%) in the ARC, suggesting the presence of KNDy neurons in the ARC of guinea pigs. In conclusion, the present study shows that SB223412 could be a candidate compound to suppress testosterone secretion in male guinea pigs for controlling sexual and aggressive behaviors in the species.

1. Introduction

The guinea pig is not only a useful experimental animal but also an important zoo animal. Guinea pigs have been kept in petting zoos and are recommended for animal-assisted activities or therapy because of their small body and amicable nature. When they are kept in zoos though, their reproduction and aggressive behaviors need to be thoroughly regulated. To inhibit undesired reproduction, male and female guinea pigs should be kept separately except when required for breeding. Furthermore, since intact male guinea pigs fight each other, they need to be kept in individual cages. This aggressive behavior may injure visitors and therefore, male guinea pigs can be inappropriate for animal-assisted activities. Currently, in order to avoid these problems, surplus male guinea pigs are culled. Suppression of male sexual and aggressive behaviors is an ideal strategy to control reproduction in guinea pig colonies and to prevent injury to other animals and visitors due to these aggressive tendencies. Controlling testosterone levels is an effective way to manipulate sexual and aggressive behaviors in male guinea pigs, because the steroid is essential for these behaviors in adult male mammals. The synthesis and secretion of testicular testosterone could be controlled by manipulating pulsatile secretion of gonadotropin-releasing hormone (GnRH)/gonadotropins.

Kisspeptin, encoded by the *Kiss1* gene and found as an

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http://dx.doi.org/10.1016/j.theriogenology.2017.07.053
0093-691X/

endogenous ligand of GPR54 [1,2], is considered a master regulator for reproduction in mammals, including rodents, ruminants and primates [3]. Central and peripheral injection of kisspeptin stimulates GnRH secretion followed by gonadotropin and sex steroid release in rats and primates [4–6]. One of the action sites of kisspeptin in the brain is thought to be GnRH neurons which stimulate gonadotropin and steroid release [7] and GPR54 gene expression in GnRH neurons is known to be essential for reproduction [8–10]. Kisspeptin neurons, therefore, control testosterone secretion and subsequent male sexual/aggressive behaviors in adult male mammals, indicating that male sexual and aggressive behaviors could be suppressed by inhibiting kisspeptin release.

Kisspeptin neurons located in the hypothalamic arcuate nucleus (ARC) express neurokinin B (NKB) and dynorphin A (Dyn) in mice [11], rats [12,13], goats [14], sheep [15] and cattle [16], and thus are referred to as KNDy neurons [17]. A hypothalamic deafferentation study showed that the GnRH/LH pulse generator might be located closely to the ARC within the mediobasal hypothalamus [18]. Additionally, multiple-unit activity (MUA) recorded from the electrodes placed in the vicinity of ARC kisspeptin neurons was synchronized with the LH pulses in goats [14]. Therefore, KNDy neurons are considered to be responsible for GnRH/luteinizing hormone (LH) pulse generation [19].

Neurokinin B and neurokinin-3 receptor (NK3R), a receptor for NKB, are widely distributed in the central and peripheral nervous system in mammals [20,21]. A loss-of-function mutation in the genes encoding NKB (*Tac3*) or NK3R (*Tacr3*) induces hypogonadotropic hypogonadism, resulting in the lack of pubertal onset, gonadotropin deficiency and infertility in humans [22–24]. The specific destruction of NK3R-expressing neurons in the ARC reduces LH secretion in rats [25,26]. These reports clearly show the significance of NKB-NK3R signaling in regulating GnRH/LH release in mammals. A previous study reported that *Tacr3* mRNA is expressed in KNDy neurons of mice [27]. We have previously shown that the oral administration of an NK3R-selective antagonist, SB223412, suppresses plasma LH and testosterone concentrations at a low level in male dogs [28]. Intravenous or oral administration of another NK3R-selective antagonist, ESN364, decreased LH levels in ewes and monkeys [29]. In humans, oral administration of ESN364 suppressed LH secretion and consequently decreased estradiol and progesterone in women and as well as testosterone in men [30]. Thus, it is plausible that the inhibitory effects of the antagonist on reproductive functions are associated with NKB-NK3R signaling in KNDy neurons, and that antagonism of signaling by SB223412 could suppress reproductive functions in male guinea pigs as well. However, SB223412 administration failed to suppress LH secretion in rats [31], suggesting that the effect of the NK3R antagonist on LH or steroids secretion is inconsistent between species. Thus, guinea pigs themselves should be examined directly to determine the effect of SB223412 on reproductive hormones in guinea pigs.

In the present study, we examined the effects of peripheral and oral administration of SB223412, an NK3R antagonist, on plasma testosterone levels in male guinea pigs to evaluate the possibility of SB223412 as a contraceptive for male guinea pigs. Furthermore, since the presence of KNDy neurons has not been examined in guinea pigs, we clarified the coexpression of kisspeptin/NKB and kisspeptin/Dyn by dual-labeling *in situ* hybridization in the ARC of male guinea pigs.

2. Materials and methods

2.1. Animals and treatments

Adult Hartley male guinea pigs (10–13 weeks of age, 500–700 g body weight) were used. All animals were housed in individual cages under a 14:10 h light/dark cycle (lights on 0500 h) at 21 ± 3 °C and provided with standard guinea pig feed (CG-7, CLEA, Tokyo, Japan) and water *ad libitum*. Intravenous cannulation was performed with inhalational anesthesia using isoflurane (Wako Pure Chemical Industries, Osaka, Japan) a day prior to blood sampling. All procedures for animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agricultural and Life Sciences, The University of Tokyo.

2.2. Single subcutaneous administration of SB223412 and blood sampling

SB223412 was purchased from AstaTech (Chengdu) Biopharmaceutical Corp in China. SB223412 (50 mg/body) dissolved in 200 μl of dimethyl sulfoxide (Wako Pure Chemical Industries) was subcutaneously administered after the first blood sampling. This dose was chosen, because a previous study showed that peripheral administration of SB223412 at 100 mg/kg body weight blocked most [^{3}H]senktide bindings to NK3R in the guinea pig brain slices [32]. Blood samples (100 μl) were collected from arterial cannula every 1 h from the 1st to 10th hour after administration. The blood samples were centrifuged (15,000×*g*, 10 min, 4 °C) and the plasma samples were stored at −30 °C until assayed for testosterone and SB223412.

2.3. Oral administration of SB223412 and blood sampling

Standard guinea pig chow was powdered by a blender, and then mixed with SB223412. The drug-containing feeds were given once a day at 1100 h at doses of 5 or 50 mg/body/day for 7 days. This higher dose was determined by considering the body-weight ratio according to the previous study using dogs [28]. Animals in the control group were fed in the same way but without SB223412. Blood samples (100 μl) were collected every day at 1100 h and centrifuged (15,000×*g*, 10 min, 4 °C). Plasma samples were stored at −30 °C until assayed for testosterone and SB223412.

2.4. Assays

Plasma testosterone concentration was measured by an enzyme immunoassay as described in previously [33]. Testosterone was extracted from 10 to 20 μl plasma samples using a mixture of hexane and ether. Rabbit anti-sheep IgG antibody (0.5 μg/100 μl/well, #613-4128, ROCK LAND, USA) was incubated for 2 h at room temperature in advance. Extracted samples and sheep anti-testosterone antibody (1:60,000, GDN #250, kindly donated by Dr. GD Niswander, Colorado University, CO, USA) were aliquoted to each well and incubated overnight at 4 °C. Then, horseradish peroxidase (HRP)-labeled testosterone, which was prepared according to a previous method [34] using testosterone 3-(*O*-carboxymethyl)oxime (T8390, Sigma Aldrich, Tokyo, Japan) and HRP (814,393, Boehringer Mannheim GmbH, Mannheim, Germany), was added to each well for a second incubation for 4 h at 4 °C. Horseradish peroxidase activity was visualized by 3, 3′, 5, 5′-tetramethylbenzidine substrate. The absorbance of each reactant was measured at 450 nm using a microplate reader. The least detectable level was 0.125 ng/ml, and intra- and inter-assay of coefficients of variation were 2.6% and 13.3% at 5.08 ng/ml, respectively.

To measure SB223412 concentrations, plasma samples (100 μl) were mixed with 500 μl of acetonitrile. The supernatant of the mixture was concentrated after centrifugation. The residue was dissolved in 100 μl of acetonitrile containing 0.01% *m*-cresol. Sample solutions were analyzed by 2996 PDA Detector (Nihon