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7α-Hydroxypregnenolone regulates diurnal changes in sexual behavior of male quail

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ABSTRACT

In the Japanese quail, 7α-hydroxypregnenolone, a previously undescribed avian neurosteroid, is actively produced in the brain. 7α-Hydroxypregnenolone acts as a novel neuronal activator to stimulate locomotor activity of quail. Therefore, in this study, we determined whether 7α-hydroxypregnenolone changes the expression of sexual behavior in Japanese quail. We first measured diurnal changes in sexual behavior of male quail exposed to a long-day photoperiod. We found that sexual behavior of male quail was high in the morning when endogenous 7α-hydroxypregnenolone level is high. Subsequently, we centrally administered 7α-hydroxypregnenolone in the evening when endogenous 7α-hydroxypregnenolone level is low. In the 30 min after intracerebroventricular (ICV) injection, 7α-hydroxypregnenolone dose dependently increased the frequency of sexual behavior of male quail. However, 7β-hydroxypregnenolone, a stereoisomer of 7α-hydroxypregnenolone, did not effect on the frequency of sexual behavior of male quail. In addition, to confirm the action of 7α-hydroxypregnenolone on sexual behavior, male birds received an ICV injection of ketoconazole, an inhibitor of cytochrome P450s, and behavioral experiments were performed in the morning. Ketoconazole significantly decreased the frequency of sexual behavior of male quail, whereas administration of 7α-hydroxypregnenolone to ketoconazole-treated males increased the frequency of their sexual behavior. These results indicate that 7α-hydroxypregnenolone regulates diurnal changes in sexual behavior of male quail.

1. Introduction

It is well established that the regulation of a variety of male reproductive behaviors, such as sexual and aggressive behaviors, are dependent of gonadal sex steroids in birds. Behavioral studies combined with endocrinological methods showed that sex steroids released from the testis, particularly testosterone, are essential for the induction of the expression of male reproductive behaviors in several species of birds, including the Japanese quail (Balthazart, 1983; Harding, 1983; Moore, 1984; Wingfield, 1985; Tsutsui and Ishii, 1981; Silver et al., 1979). For example, castration of adult males leads to decreases or losses of sexual and aggressive behaviors, and replacement therapy with testosterone restores these behaviors in birds (Adkins and Adler, 1972; Tsutsui and Ishii, 1981; Wingfield et al., 1987). One of the targets of testosterone is the preoptic and hypothalamic neurons, because testosterone and/or its metabolite-concentrating cells are present in several preoptic and hypothalamic nuclei in birds (Arnold et al., 1976; Watson and Adkins-Regan, 1989; Zigmond et al., 1980). From the pioneering finding obtained by the electrical stimulation of the preoptic and hypothalamic areas, these brain regions are known to be involved in the control of male reproductive behaviors (Åkerman, 1996; Maley, 1969; Phillips and Youngren, 1971).

It is now established that steroids can be synthesized *de novo* in the central and peripheral nervous systems. Such steroids are called "neurosteroids", and *de novo* neurosteroidogenesis from cholesterol is a conserved property of the brain of vertebrates (for reviews, see Baulieu, 1997; Compagnone and Mellon, 2000; Do Rego et al., 2009; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Tsutsui et al., 1999, 2003, 2006). Our previous studies using Japanese quail have demonstrated that the avian brain possesses the key steroidogenic enzyme, cytochrome P450 side-chain cleavage enzyme (cytochrome P450scc), and produces pregnenolone, a precursor of neurosteroids (Tsutsui and Yamazaki, 1995; Tsutsui et al., 1997; Usui et al., 1995). Other steroidogenic enzymes also are expressed in the avian brain, and convert pregnenolone to progesterone, 3β,5β-tetrahydroprogesterone, androstenedione, testosterone, and estradiol (Matsunaga et al.,

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2001, 2002, 2004a; Tsutsui and Schlinger, 2001; Ukena et al., 1999, 2001). We recently found that birds, amphibians and fish actively produce 7α-hydroxypregnenolone, a previously undescribed bioactive neurosteroid (Haraguchi et al., 2009, 2010, 2012, 2015; Matsunaga et al., 2004b; Tsutsui et al., 2008) in the brain. This novel bioactive neurosteroid acts as a neuronal modulator to increase locomotor activity in these vertebrates (Hatori et al., 2011; Matsunaga et al., 2004b; Tsutsui et al., 2008). The production and concentration of 7α-hydroxypregnenolone in the male diencephalon changed markedly during the observed 24 h period, with a maximal level at 11:00 a.m. when locomotor activity of males was high in Japanese quail (Tsutsui et al., 2008, 2009a,b). Thus, 7α-hydroxypregnenolone is considered to play a crucial role in the process of diurnal changes in locomotor activity of male quail. We further investigated the expression of cytochrome P450 7α-hydroxylase (cytochrome $P450_{7\alpha}$), a steroidogenic enzyme that catalyzes the formation of 7α-hydroxypregnenolone from pregnenolone, by *in situ* hybridization to identify the cells producing 7α-hydroxypregnenolone in the quail brain. In the male diencephalon, the expression of cytochrome $P450_{7\alpha}$ mRNA was localized mainly in the nucleus preopticus medialis (POM), the nucleus paraventricularis magnocellularis (PVN) and the nucleus ventromedialis hypothalami (VMN) in the preoptic and hypothalamic areas (Tsutsui et al., 2008). This finding suggests that 7α-hydroxypregnenolone may take part in the induction of some reproductive behaviors, such as sexual and aggressive behaviors, in birds.

The present study was designed to determine the functional role of 7α-hydroxypregnenolone in sexual behavior using Japanese quail. We first measured diurnal changes in sexual behavior of male quail. We previously found that locomotor activity of male birds exposed to a long-day photoperiod are high in the morning when endogenous 7α-hydroxypregnenolone level is high (Tsutsui et al., 2008). In the present study, we centrally administered 7α-hydroxypregnenolone or 7β-hydroxypregnenolone, a stereoisomer of 7α-hydroxypregnenolone, to male birds exposed to a long-day photoperiod in the evening when endogenous 7α-hydr oxypregnenolone level is low (Tsutsui et al., 2008). Furthermore, male birds received an intracerebroventricular (ICV) injection of ketoconazole, an inhibitor of cytochrome P450s, and behavioral experiments were performed in the morning under a long-day photoperiod. Here, we show that 7α-hydroxypregnenolone regulates diurnal changes in sexual behavior of male quail.

2. Materials and methods

2.1. Animals

Three-month old Japanese quail, *Coturnix japonica*, were used in this study. All birds were maintained in a long-day photoperiod (16/8 h light/dark cycle; lights on at 7:00 a.m., off at 11:00 p.m.). All subjects were sexually mature as demonstrated by an enlarged cloacal gland in males and by regular egg laying in females. The size of the cloacal gland, which is closely correlated with size of the testes and plasma testosterone level, provides an external marker of circulating testosterone concentration (Sachs, 1967; Ball and Balthazart, 2010). Birds were provided with food and water *ad libitum*. They were housed in individual cages (30 cm long, 14 cm wide, 16 cm high) in one room. Experiments were approved by the Ethics Committee for the Use of Animals at Waseda University, Tokyo, Japan.

2.2. Measurement of sexual behavior

Sexual behavior was recorded as described previously (Ishii and Tsutsui, 1982; Tsutsui and Ishii, 1981; Tsutsui et al., 1985; Ubuka et al., 2014). When sexually active male and female quail are paired in a relatively small cage males display the following actions of sexual behavior: (i) chasing, (ii) pecking, (iii) head grabbing (grabbing the back of the head with their back), (iv) mounting, and (v) cloacal contact movement (CCM) (Ishii and Tsutsui, 1982; Tsutsui and Ishii, 1981; Ubuka et al., 2014). For the observation of sexual behavior of males, a test bird (male) was paired with a female in the observation cage of dimensions 35 × 27 × 25 cm. The number of each action displayed by a test bird (male) against a female was recorded for 5 min. The observation was repeated with the same female. The frequencies of five actions were obtained by the calculated total numbers of two observations. Behavioral experiments were performed during 9:00–12:00 a.m. and 5:00–8:00 p.m.

2.3. Surgery and central administration of 7α-hydroxypregnenolone

To determine the possible action of 7α-hydroxypregnenolone on male sexual behavior, surgery and ICV injection of 7α-hydroxypregnenolone were conducted in male birds. All surgery was performed under Nembutal anesthesia (40 mg/kg). Using a stereotaxic instrument, male birds were chronically implanted with a 12-mm, 23-gauge steel guide cannula (Eicom, Kyoto, Japan) aimed at the lateral ventricle of the brain. Ten days after surgery, male birds received an ICV injection of vehicle or 7α-hydroxypregnenolone *via* a 13-mm, 30-gauge stainless steel injector. 7α-Hydroxypregnenolone (Steraloids, Newport, RI) dissolved in isotonic saline containing 0.2% DMSO was injected over a period of 30 sec into the lateral ventricle at different doses (10 and 100 ng in a 5-μl solution). For 30 min after 7α-hydroxypregnenolone administration, male sexual behavior was measured. The effect of 7β-hydroxypregnenolone (Steraloids, Newport, RI), a stereoisomer of 7α-hydroxypregnenolone, on male sexual behavior was also examined. Control treatment consisted of an equal volume of vehicle alone. Behavioral experiments were performed during 5:00–8:00 p.m. when endogenous 7α-hydroxypregnenolone synthesis in the brain of intact male birds is low (Tsutsui et al., 2008). Male birds received an ICV injection of ketoconazole (5 μg in a 5-μl solution) and behavioral experiments were performed during 9:00–12:00 a.m.

2.4. Statistical analysis

Data were statistically analyzed with one-way ANOVA (when a normal distribution was found), and then Tukey-Kramer test was performed as a post hoc test. Student's *t* test was conducted to determine significance when the experiment consisted of only two groups.

3. Results

3.1. Diurnal changes in the frequency of male sexual behavior

Diurnal changes in the frequency of sexual behavior were examined in male birds. Behavioral analysis indicated that the frequency of five actions (i.e., chasing, pecking, head grabbing, mounting and CCM) of sexual behavior of male birds was much higher ($P < 0.05$) in the morning (9:00–12:00 a.m.) than the evening (5:00–8:00 p.m.) under a long-day photoperiod (16/8 h light/-dark cycle; lights on at 7:00 a.m., off at 11:00 p.m.) (Fig. 1A–E).

3.2. Stimulation of male sexual behavior by 7α-hydroxypregnenolone administration

Male birds received an ICV injection of vehicle or 7α-hydroxypregnenolone under a long-day photoperiod. Behavioral