Review

Breakthrough in neuroendocrinology by discovering novel neuropeptides and neurosteroids: 2. Discovery of neurosteroids and pineal neurosteroids

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ABSTRACT

Bargmann–Scharrer’s discovery of “neurosecretion” in the first half of the 20th century has since matured into the scientific discipline of neuroendocrinology. Identification of novel neurohormones, such as neuropeptides and neurosteroids, is essential for the progress of neuroendocrinology. Our studies over the past two decades have significantly broadened the horizons of this field of research by identifying novel neuropeptides and neurosteroids in vertebrates that have opened new lines of scientific investigation in neuroendocrinology. We have established de novo synthesis and functions of neurosteroids in the brain of various vertebrates. Recently, we discovered 7α-hydroxypregnenolone (7α-OH PREG), a novel bioactive neurosteroid that acts as a key regulator for inducing locomotor behavior by means of the dopaminergic system. We further discovered that the pineal gland, an endocrine organ located close to the brain, is an important site of production of neurosteroids and its contribution to the progress of neuroendocrinology.

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1. Introduction

Scharrer proposed the concept of “neurosecretion” that neurons secrete hormones in the 1920s, and it was established by Bargmann in 1949. The discovery of “neurosecretion” created neuroendocrinology, a new research field in endocrinology and neuroscience. Identification of novel neurohormones that regulate physiological processes is essential for the progress of neuroendocrinology. Past studies have significantly broadened the horizons of this field of research by identifying novel neuropeptides and neurosteroids in the brain of vertebrates that have opened new lines of scientific investigation in neuroendocrinology.

The brain has traditionally been considered as a target site for peripheral steroid hormones in vertebrates. Thirty years ago, however, Baulieu and colleagues discovered that certain steroid hormones are present in higher amounts in the brain than in the plasma in mammals (for a review, Baulieu, 1997). They also found that in mammals suppression of circulating steroids by removing gonads and adrenal glands does not affect the concentrations of pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfate esters in the brain (for a review, Baulieu, 1997). Extensive studies over the past two decades have demonstrated that the central and peripheral nervous systems have the capacity of synthesizing steroids from cholesterol (CHOL), the so-called “neurosteroids” (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, 2000, 2003, 2006). De novo formation of neurosteroids in the brain was originally demonstrated in mammals (Compagnone et al., 1995; Corpéchot et al., 1981, 1983; Jo et al., 1989; Lanthier and Patwardhan, 1986; Mathur et al., 1993; Mellon and Deschepper, 1993; Robel and Baulieu, 1985; Robel et al., 1987), and subsequently in birds (Freking et al., 2000; London and Schlinger, 2007; London et al., 2003, 2006, 2010; Matsunaga et al., 2001, 2002; Schlinger et al., 1999; Soma et al., 2004; Tam and Schlinger, 2007; Tsutsui and Schlinger, 2001; Tsutsui and Yamazaki, 1995; Tsutsui et al., 1997, 1999, 2003; Ukena et al., 1999, 2001; Usui et al., 1995; Vanson et al., 1996), amphibians (Beaujean et al., 1999; Bruzzone et al.,...
2. Discovery of 7α-OH PREG in the brain as a new regulator of locomotor behavior

2.1. Background

It is well established that the central and peripheral nervous systems synthesize neurosteroids de novo from CHOL (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, 2000, 2003, 2006). The formation of neurosteroids in the brain was originally demonstrated in mammals by Baulieu and colleagues (Compagnone et al., 1995; Corpéchot et al., 1981, 1983; Jo et al., 1989; Lanthier and Patwardhan, 1986; Mathur et al., 1993; Mellon and Deschepper, 1993; Robel and Baulieu, 1983; Robel et al., 1987). The brain of non-mammalian vertebrates also produces a variety of neurosteroids (for reviews, see Do-Rego et al., 2009; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Tsutsui et al., 1999, 2000, 2003, 2006). In birds, the formation of neurosteroids in the brain has been demonstrated both in galliform bird species such as the Japanese quail (Matsunaga et al., 2001, 2002; Tsutsui and Schlinger, 2001; Tsutsui and Yamazaki, 1995; Tsutsui et al., 1997, 1999, 2003; Ukena et al., 1999, 2001; Usui et al., 1995) and in passeriform bird species such as the zebra finch (Freking et al., 2000; London and Schlinger, 2007; London et al., 2003, 2006, 2010; Schlinger et al., 1999; Soma et al., 2004; Tam and Schlinger, 2007; Vanson et al., 1996). The formation of neurosteroids from CHOL is now also well documented in various species of amphibians (Beaujane et al., 1999; Bruzzone et al., 2010; Do-Rego et al., 2007; Inai et al., 2003; Matsunaga et al., 2004a; Mensah-Nyagan et al., 1994, 1996a,b,1999; Takase et al., 1999, 2002, 2011) and fish (Brion et al., 2012; Diotel et al., 2011; Menuet et al., 2005; Sakamoto et al., 2001). Therefore, de novo synthesis of neurosteroids from CHOL in the brain appears to be conserved across vertebrate species (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, 2000, 2003, 2006). However, the biosynthetic pathways leading to the formation of neurosteroids in the brain are still not completely elucidated in vertebrates (for a review, Tsutsui et al., 2006). In fact, Tsutsui and colleagues recently identified 7α-hydroxyprogrenolone (7α-OH PREG) as a novel bioactive neurosteroid stimulating locomotor behavior in the brain of quail (Tsutsui et al., 2008) and Japanese red-bellied newts Cynops pyrrhogaster (Matsunaga et al., 2004a). It was also found that cytochrome P450 7α-hydroxylase (cytochrome P450c51; gene name Cyp7b) catalyzes PREG to produce 7α-OH PREG in the brain of these species (Haraguchi et al., 2010; Tsutsui et al., 2008). The regulation of biosynthesis, mode of action and functional significance of this novel bioactive neurosteroid are now becoming clear.

Until recently, it was generally believed that neurosteroids are produced in neurons and glial cells in the central and peripheral nervous systems (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, 2000, 2003, 2006). However, Tsutsui and colleagues recently discovered that the pineal gland, an endocrine organ located close to the brain, also produces a variety of neurosteroids (for reviews, see Mellon and Deschepper, 1993; Robel and Baulieu, 1997; Compagnone and Mellon, 2000; Do-Rego et al., 2009; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Tsutsui et al., 1999, 2000, 2003, 2006). Therefore, the pineal gland produces 7α-OH PREG on locomotor behavior has thus been tested in the subphylum vertebrata (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, 2000, 2003, 2006). However, the biosynthetic pathways leading to the formation of neurosteroids in the brain are still incompletely elucidated in vertebrates (for a review, see Tsutsui et al., 2006).

2.2. Discovery of 7α-OH PREG in the brain as a new regulator of locomotor behavior

Tsutsui and colleagues recently discovered that the amphibian brain actively produces a previously undescribed neurosteroid, 7α-OH PREG through α-hydroxylation of PREG at the 7-position, based on biochemical techniques combined with high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and gas chromatography-mass spectrometry (GC–MS) analyses using the Japanese red-bellied newt as an animal model to identify this novel amphibian neurosteroid (Fig. 1; Matsunaga et al., 2004a). The same biochemical and analytical approaches later demonstrated that the brain of the Japanese quail also produces 7α- and 7β-OH PREG (Tsutsui et al., 2008).

In both amphibians and birds, cytochrome P450c51 catalyzes the conversion of PREG into 7α-OH PREG in the brain (Fig. 1; Haraguchi et al., 2010; Tsutsui et al., 2008). A 2598-bq cDNA encoding a putative cytochrome P450c51 has been identified from the newt brain (Haraguchi et al., 2010). The enzymatic activity of this putative new P450c51 was demonstrated biochemically (Haraguchi et al., 2010). Combination of HPLC and GC–MS analyses revealed that the homogenate of COS-7 cells transfected with the putative new P450c51 cDNA produced 7α-OH PREG from PREG. A 2341-bp full-length cDNA prepared from the quail brain was also identified as encoding a putative cytochrome P450c51 (Tsutsui et al., 2008). The homogenate of CO2–7 cells transfected with the putative quail P450c51 cDNA converted PREG into 7α-OH PREG (Tsutsui et al., 2008). Although both 7α- and 7β-OH PREG are present in the quail brain (Tsutsui et al., 2008), it is still unclear whether cytochrome P450c51 can also convert PREG into 7β-OH PREG in birds. Because the presence of 7α-OH PREG and cytochrome P450c51 has also been identified in the brain of mammals (Awka et al., 1992; Doostzadeh and Morfin, 1997; Weill-Engerer et al., 2003; Yau et al., 2003) and fish (Haraguchi, S., Suzuki, Y., Ueda, H., Tsutsui, K., unpublished observation), the production of 7α-OH PREG in the brain is considered to be a common feature in vertebrates.

Because 7α-OH PREG is actively synthesized in the brain of newts, this amphibian species has been used as an animal model to investigate the biological action of 7α-OH PREG. The effect of 7α-OH PREG on locomotor behavior has thus been tested in male newts (Matsunaga et al., 2004a). Intracerebroventricular (ICV) injection of 7α-OH PREG markedly increased walking and
swimming movements of male newts within the next 30-min (Fig. 1; Matsunaga et al., 2004a). The stimulatory effect of 7α-OH PREG was dose-dependent, corresponding to the physiological range observed in the brain of normal male newts (Fig. 1; Matsunaga et al., 2004a). Since the quail displays a robust locomotor rhythm when held under typical light/dark lighting schemes (Wada, 1979; Wilson, 1972), this avian species has also proven to be an appropriate animal model to investigate the biological action of 7α- and 7β-OH PREG. ICV injection of 7α-OH PREG to male quail also produced a dose-dependent stimulation of locomotor behavior (Tsutsui et al., 2008). In contrast, 7β-OH PREG did not influence locomotor behavior (Tsutsui et al., 2008). It thus appears that 7α-OH PREG acts to stimulate locomotor behavior in male newts and quail (for reviews, see Haraguchi et al., 2011; Tsutsui et al., 2009a,b, 2010a,b, 2012, 2013). Studies in Cyp7b gene-deficient mice are now in progress to clarify the biological action of 7α-OH PREG on locomotion in mammals.

2.3. Mode of action of 7α-OH PREG in the brain

Based on a series of experiments using the male newt, Tsutsui and colleagues have indicated that 7α-OH PREG acts as a neuronal modulator to stimulate locomotor behavior through the dopaminergic system (Fig. 2). 7α-OH PREG increased the concentration of dopamine in the male newt brain, especially in the rostral brain region including the striatum, which is known to be involved in the regulation of locomotor behavior (Matsunaga et al., 2004a). 7α-OH PREG also increased dopamine release from cultured male brain in vitro (Matsunaga et al., 2004a). The effect of 7α-OH PREG on locomotor behavior was abolished by administration of the dopamine D2 receptor antagonists, haloperidol and sulpiride (Matsunaga et al., 2004a). To recapitulate, 7α-OH PREG synthesized in the newt diencephalon and rhombencephalon, by acting on dopaminergic neurons localized in the posterior tuberal nucleus (PT) and ventral tegmental area (VTA), may induce dopamine release from

Fig. 1. Identification of 7α-OH PREG in the brain. Newts in the spring breeding period (April–May) were used. (A) Reversed-phase HPLC analysis showing the formation of an unknown metabolite of PREG after incubation of newt brain homogenates with 3H-PREG at 25°C. The steroids were eluted with a 30-min linear gradient of 40–70% acetonitrile at a flow rate of 0.7 ml/min, followed by an isocratic elution of 70% acetonitrile. The ordinate indicates the radioactivity measured in each HPLC fraction, and the arrows indicate the elution positions of standard steroids, i.e. 7α- and 7β-OH PREG, and PREG. (B) TLC analysis of the unknown PREG metabolite and standard steroids 7α- and 7β-OH PREG. The unknown PREG metabolite was detected by autoradiography. Standard steroids 7α- and 7β-OH PREG were visualized by iodine vapors. (C) GC–MS of trimethylsilyl ether derivatives of the unknown PREG metabolite and authentic 7α-OH PREG. (D) Biosynthetic pathways for the formation of 7α-OH PREG in the brain. Cytochrome P450scc converts PREG to 7α-OH PREG. P450scc, cytochrome P450 side-chain cleavage enzyme (gene name Cyp11a); P450α2, cytochrome P450 7α-hydroxylase (gene name Cyp7b). (E) Effect of 7α-OH PREG on locomotor behavior in the male newt. Male newt received an ICV injection of vehicle (n = 7) or 7α-OH PREG (0.1, 0.5 or 1 ng: n = 6 or 7). For behavioral testing, newts were placed individually in a water-filled aquarium maintained; each testing arena was marked with parallel lines. Immediately after administration of 7α-OH PREG, locomotor behavior was quantified by counting the total number of lines crossed during a 30-min observation. Locomotor behavior consisted of a combination of walking and swimming movements. Each column and vertical line represent the mean ± SEM. *P < 0.01 vs. vehicle, †P < 0.05 vs. 0.1 ng 7α-OH PREG injection by one-way ANOVA, followed by Duncan’s multiple range test. See Haraguchi et al. (2010), Matsunaga et al. (2004a) and the text for details.
can also act of 3 via dopamine release (Tsutsui et al., 2010, 2012b, Matsunaga et al., 2004a, Tsutsui et al., 2008) and/or NMDA receptors, or through an unknown membrane receptor. In birds (Ball et al., 1995; Levens et al., 2000; Hara et al., 2007; Mezey and Csillag, 2002; Acosta et al., 2000), PREG in the diencephalon may regulate the biosynthesis of 7α-OH PREG in the brain. See Haraguchi et al., 2010, 2012b, Matsunaga et al., 2004a, Tsutsui et al., 2008 and the text for details.

Fig. 2. Mode of action and regulation of biosynthesis of 7α-OH PREG in the brain. Schematic models depicting the mode of action of 7α-OH PREG on the regulation of locomotor behavior, and the action of melatonin, prolactin (PRL) and corticosterone (CORT) on the regulation of biosynthesis of 7α-OH PREG in the brain. See Haraguchi et al., 2010, 2012b, Matsunaga et al., 2004a, Tsutsui et al., 2008 and the text for details.

their terminals in the rostral brain region, notably in the striatum and nucleus accumbens (NA), and consequently increase locomotor behavior of male newts (Fig. 2; Matsunaga et al., 2004a).

In male quail, the expression of P450<sub>27α</sub> mRNA was localized in several diencephalic regions, such as the nucleus preopticus medialis (POM), the paraventricular nucleus (PVN), the nucleus ventromedialis hypothalami (VMM), the nucleus dorsolateralis anterior thalami (DLA), and the nucleus lateralis anterior thalami (LA) (Tsutsui et al., 2008). Dopaminergic neurons that are located in the mesencephalic region, including the VTA and the substantia nigra (SN), project to the telencephalic region, in particular in the striatum in birds (Hara et al., 2007; Mezey and Csillag, 2002). The telencephalic region is enriched with dopamine D<sub>1</sub> and D<sub>2</sub> receptors in birds (Ball et al., 1995; Levens et al., 2000). Accordingly, 7α-OH PREG actively synthesized in the diencephalon may act on dopamine neurons localized in the VTA and SN in the mesencephalon to stimulate dopamine release from their terminals in the striatum, and increase locomotor behavior in male quail as in male newts (for reviews, see Haraguchi et al., 2011; Tsutsui et al., 2009a,b, 2010a,b, 2012, 2013).

Because 7α-OH PREG stimulates acutely locomotor behavior in newts (Matsunaga et al., 2004a) and quail (Tsutsui et al., 2008), this neurosteroid may act through a non-genomic rather than a genomic mechanism. It has been reported that 3α,5α-THP, a progesterone metabolite, modulates locomotion (Wieland et al., 1995) and dopamine release (Bullock et al., 1997; Rougé-Pont et al., 2002) via a non-genomic pathway in rats. The neuremodulatory action of 3α,5α-THP may be mediated through type A γ-aminobutyric acid (GABA<sub>A</sub>) receptor, since 3α,5α-THP is a potent allosteric modulator of GABA<sub>A</sub> receptor (Lambert et al., 1995; Paul and Purdy, 1992) and dopaminergic neurons are regulated by GABAergic transmission (Laviolette and van der Kooy, 2001). Similarly, PREG can also act via non-genomic mechanisms by binding to GABA<sub>A</sub> and N-methyl-D-aspartate (NMDA) receptors to enhance neuronal excitability (Paul and Purdy, 1992; Sliwinski et al., 2004). Whether the acute actions of 7α-OH PREG on dopamine release and locomotor behavior in newts and quail are also mediated through GABA<sub>A</sub> and/or NMDA receptors, or through an unknown membrane receptor, remains to be determined.

2.4. Functional significance and regulation of biosynthesis of 7α-OH PREG in the brain

It is important to clarify the functional significance of 7α-OH PREG in the regulation of locomotor behavior. Tsutsui and colleagues therefore investigated diurnal changes in both locomotor behavior and diencephalic 7α-OH PREG concentrations in the male quail exposed to a daily photoperiod of 16/8 h light/dark cycles (LD; lights on at 07:00 am, off at 11:00 pm). Locomotor behavior of males was much higher than that of females from the time of lights on until noon, but decreased to female levels thereafter (Tsutsui et al., 2008). These changes in locomotor behavior in males were directly correlated with 7α-OH PREG concentrations in the diencephalon (Tsutsui et al., 2008). Furthermore, administration of ketoconazole, an inhibitor of cytochrome P450s, to males suppressed locomotor behavior (Tsutsui et al., 2008). Thus, the increase in 7α-OH PREG in the diencephalon may account for the higher locomotor behavior in males. In females, the lower level of 7α-OH PREG in the diencephalon suggests that this neurosteroid may not be involved in the control of locomotor behavior (Tsutsui et al., 2008).

Because melatonin is known to be involved in the regulation of locomotor behavior in birds (Binkley et al., 1971; Cassone and Menaker, 1984; Chabot and Menaker, 1992; Hau and Gwinner, 1994; John et al., 1978; Murakami et al., 2001; Warren and Cascone, 1995), Tsutsui and colleagues hypothesized that melatonin may regulate the biosynthesis of 7α-OH PREG in the diencephalon, and thereby influence locomotor behavior. Pinealectomy (Px) and orbital enucleation (Ex) were carried out to investigate the possible involvement of melatonin in the regulation of diurnal changes in 7α-OH PREG production in male quail (Tsutsui et al., 2008). Concomitant Px and Ex increased the production and concentration of 7α-OH PREG and the expression of P450<sub>27α</sub> mRNA in the diencephalon (Tsutsui et al., 2008). Reciprocally, melatonin administration to Px × Ex birds decreased the production and concentration of 7α-OH PREG and the expression of P450<sub>27α</sub> mRNA in the diencephalon (Tsutsui et al., 2008). The inhibitory effect of melatonin on 7α-OH PREG synthesis was abolished by luzindole, a melatonin receptor antagonist (Tsutsui et al., 2008). It is therefore considered that melatonin secreted by the pineal gland and eyes acts as an inhibitory regulator of 7α-OH PREG biosynthesis in the quail brain (Fig. 2). This mechanism is in harmony with earlier studies indicating that melatonin treatment reduces locomotor behavior in quail (Murakami et al., 2001; Nakahara et al., 2003), sparrow (Murakami et al., 2001) and owl (Murakami et al., 2001). There is a circadian locomotor rhythm controlled by daily rhythm of melatonin secretion in birds and other vertebrates (Binkley et al., 1971; Cassone and Menaker, 1984; Chabot and Menaker, 1992; Hau and Gwinner, 1994; John et al., 1978; Murakami et al., 2001; Saper et al., 2005; Warren and Cassone, 1995). Because 7α-OH PREG is also produced in the brain of newts (Matsunaga et al., 2004a), mammals (Akwa et al., 1992; Doostzadeh and Morfin, 1997; Weil-Engerer et al., 2003; Yau et al., 2003), and fish (Haraguchi, S., Suzuki, Y., Ueda, H., Tsutsui, K., unpublished observation), a similar mechanism may be evident in these vertebrates (for reviews, see Tsutsui et al., 2009a,b, 2010a,b, 2012, 2013).

To further understand the functional significance and regulation of biosynthesis of 7α-OH PREG, seasonal changes in this neurosteroid in the brain have been investigated in newts (Haraguchi et al., 2010; Matsunaga et al., 2004a). Marked changes in the production and concentration of 7α-OH PREG were observed in the male brain during the annual breeding cycle with higher levels in the spring breeding period (Haraguchi et al., 2009, 2010; Matsunaga et al., 2004a). The expression of P450<sub>27α</sub> mRNA in the male brain showed similar seasonal changes (Haraguchi et al., 2010). These observations support the view that the rise in locomotor behavior in male newts during the spring breeding period can be ascribed to an increase in 7α-OH PREG synthesis in the brain. In contrast to males, 7α-OH PREG levels in the brain of females did not vary significantly and were constantly low (Haraguchi et al., 2010). Thus, the slower locomotion in females...
may be accounted for by a lower level of 7α-OH PREG in their brain.

It is known that in the male newt plasma prolactin (PRL) and gonadotropin (GTH) concentrations increase during the breeding season (Kano et al., 2005; Matsuda et al., 1990; Mosconi et al., 1994). To determine the possible involvement of PRL or GTH in the regulation of seasonal changes in 7α-OH PREG synthesis in the brain, hypophysectomy (Hypox) and IVC injection of each adenohypophyseal hormone were conducted in male newts in the spring breeding period. The production and concentration of 7α-OH PREG in the brain of sexually mature males decreased within two weeks after Hypox, suggesting the involvement of PRL or GTH rather than melatonin in the regulation of seasonal changes in 7α-OH PREG synthesis in the brain (Haraguchi et al., 2010). Administration of PRL, but not GTH to Hypox male newts restored the production and concentration of 7α-OH PREG in the brain (Haraguchi et al., 2010). Inversely, administration of anti-newt PRL antibodies decreased 7α-OH PREG production (Haraguchi et al., 2010). Thus, these data indicate that PRL secreted by the adenohypophysis is involved in the regulation of 7α-OH PREG biosynthesis (Fig. 2). This is an unprecedented action of adenohypophyseal hormone in the regulation of neurosteroidogenesis in the brain of any vertebrate.

In contrast to male newts, no seasonal changes in the production and concentration of 7α-OH PREG and the expression of P450\(_{17\alpha}\) mRNA occurred in female newts (Haraguchi et al., 2010). It is known in newts that plasma PRL levels in females are constantly low during the annual breeding cycle unlike in males (Matsuda et al., 1990). Such a sexual dimorphism in the seasonal changes in plasma PRL levels may account for the absence of seasonal changes in 7α-OH PREG synthesis in the female brain.

To explore the mode of action of PRL in the regulation of 7α-OH PREG biosynthesis, the localization of cytochrome P450\(_{17\alpha}\) and PRL receptor (PRLR) was investigated in sexually mature male newts (Haraguchi et al., 2010). P450\(_{17\alpha}\)-positive neurons were localized mainly in the anterior preoptic area (POA), magnocellular preoptic nucleus (Mg), and tegmental area (TA) in the brain (Haraguchi et al., 2010). However, PRLR-like immunoreactivity was only detected in the Mg (Haraguchi et al., 2010). Thus, the major targets of PRL action to increase 7α-OH PREG biosynthesis may be the P450\(_{17\alpha}\)-positive neurons in the Mg. The Mg is sexually dimorphic both in terms of neuroanatomical structure and response to pheromones (Govek and Swann, 2007). In particular, the Mg possesses a higher number of neurons in the male than in the female (Govek et al., 2003). Electrotokyic lesions of the Mg suppress male copulatory behavior in the hamster (Govek et al., 2003). PRL acts centrally to induce courtship behavior in male newt (Giorgio et al., 1982; Toyoda et al., 1993). Since 7α-OH PREG also enhances sexual behavior in male newts (Toyoda et al., 2012), PRL may stimulate locomotor behavior and courtship behavior by increasing 7α-OH PREG synthesis in the Mg of sexually mature male newts (for reviews, see Haraguchi et al., 2011; Tsutsui et al., 2009a, 2010a, b, 2012). It is known that acute stress affects locomotor behavior in vertebrates (Hubbard et al., 2010; Lee et al., 1986). Stress stimulates the hypothalamo-pituitary-adrenal axis leading to an increase in the plasma glucocorticoid (GC) level. There is strong evidence that GC administration provokes marked changes in locomotor behavior (Breuner et al., 1998; Breuner and Wingfield, 2000; Mitra and Sapolsky, 2008; Moore and Miller, 1984; Ricciardella et al., 2010). However, the molecular mechanisms mediating the effect of GC on locomotor behavior were poorly understood. Based on the stimulatory action of 7α-OH PREG on locomotion, Tsutsui and colleagues hypothesized that elevated GC concentrations under acute stress may increase 7α-OH PREG biosynthesis in the brain, and consequently stimulate locomotor behavior. To test this hypothesis, Tsutsui and colleagues investigated the effects of acute stress on the production and concentration of 7α-OH PREG in male newts. A 30-min restraint stress increased the plasma corticosterone (CORT) level, stimulated the production of 7α-OH PREG and elevated the concentration of this neurosteroid in the brain of male newts (Haraguchi et al., 2012b). A 30-min restraint stress also increased the expression of cytochrome P450\(_{17\alpha}\) in the dorsomedial hypothalamus (DMH) (Haraguchi et al., 2012b). Decreasing plasma CORT concentrations by Hypox decreased 7α-OH PREG synthesis, whereas administration of CORT to Hypox newts increased 7α-OH PREG synthesis (Haraguchi et al., 2012b).

In vertebrates, the DMH plays an important role in coordinating physiological and behavioral responses to stress (DiMicco et al., 2002; Lowry et al., 2001, 2003). Serotonin is known to be implicated in the control of locomotor behavior in vertebrates (Li et al., 2004; Lorrain et al., 1997, 1999; Takahashi et al., 2000). Tsutsui and colleagues recently provided new evidence that the pineal gland is a major neuroendocrine system for the past thirty years (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;
2000, 2003, 2006). Molecular and biochemical techniques have further demonstrated that 7α-OH PREG and 3α,5α-THP are major neurosteroids secreted by the pineal gland (Haraguchi et al., 2012a; Hatori et al., 2011). The biological actions of pineal 7α-OH PREG and 3α,5α-THP are now becoming clear (Haraguchi et al., 2012a; Hatori et al., 2011).

3.2. Discovery of neurosteroidogenesis in the pineal gland

Recently, Tsutsui and colleagues discovered that the pineal gland actively synthesizes neurosteroids de novo from CHOL via PREG in chickens (Hatori et al., 2011) and quail (Fig. 3; Haraguchi et al., 2012a). PREG is known to be the common precursor of all steroid hormones in vertebrates and the formation of PREG is initiated by cleavage of the side-chain of CHOL by cytochrome P450sc5c (P450sc5c; gene name Cyp11a), a rate-limiting mitochondrial enzyme originally found in peripheral steroidogenic organs. The demonstration of PREG formation from CHOL is therefore essential to establish de novo neurosteroidogenesis in the pineal gland. Tsutsui and colleagues first found that cytochrome P450sc5c is expressed in the pineal gland of juvenile chickens (Hatori et al., 2011) and juvenile quail (Haraguchi et al., 2012a) by reverse transcription polymerase chain reaction (RT-PCR) analysis. Immunohistochemical analysis using cytochrome P450sc5c antibodies showed intense staining in the cells forming follicular structures in the quail pineal gland (Haraguchi et al., 2012a). Incubation of the pineal glands from quail chicks with 1H-CHOL formed a radioactive metabolite that, upon HPLC analysis, exhibited the same retention time as PREG (Haraguchi et al., 2012a). GC–MS analysis further demonstrated the occurrence of PREG in the pineal gland (Haraguchi et al., 2012a).

Subsequently, the expressions of several key steroidogenic enzymes, including cytochrome P450 7α-hydroxylase (cytochrome P450sc5; gene name Cyp7b), 3α-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3α-HSD; gene name Hsd3a), 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD; gene name Hsd3b), 5α-reductase (gene name Srd5a), 5β-reductase (gene name Srd5b), cytochrome P450 17α-hydroxylase/c17,20-lyase (P450c17,20lyase; gene name Cyp17), 17β-hydroxysteroid dehydrogenase (17β-HSD; gene name Hsd17b) and cytochrome P450 aromatase (P450arom; gene name Cyp19) have been demonstrated in the pineal gland of juvenile chickens and quail by RT-PCR analyses (Haraguchi et al., 2012a; Hatori et al., 2011). To clarify the biosynthetic pathways of neurosteroids in the pineal gland, biochemical studies combined with HPLC and GC–MS analyses were further conducted. Pineal gland homogenates from quail chicks were incubated with 3H-PREG as a precursor and, subsequent analysis of the products by reversed-phase HPLC detected the formation of 7α- and/or 7β-OH PREG (Fig. 3; Haraguchi et al., 2012a). In addition to these neurosteroids, progesterone (PREG), 3α,5α-THP and 3α,5β-THP, androstenedione (AD), testosterone (T), 5α- and/or 5β-dihydrotestosterone (5α- and/or 5β-DHT) and estradiol-17β (E2) were produced from the precursor PREG (Fig. 3; Haraguchi et al., 2012a). Although isomers, such as 7α- and 7β-OH PREG; 3α,5α- and 3β,5β-THP; and 5α- and 5β-DHT, were not separated from each other by HPLC analysis, the formation of these neurosteroids in the pineal gland was demonstrated by GC–MS analysis (Haraguchi et al., 2012a). Derivatives of synthetic 7α- and 7β-OH PREG, PROG, 3α,5α- and 3β,5β-THP, AD, T, 5α- and 5β-DHT, E2, and the purified nonradioactive steroids produced by the pineal gland were applied to GC-selected ion monitoring (SIM) analysis, which showed the same mass spectral characteristics: mass/charge (m/z) 386 for 7α- and 7β-OH PREG, m/z 510 for PROG, m/z 514 for 3α,5α- and 3β,5β-THP, m/z 482 for AD, m/z 680 for T, m/z 486 for 5α- and 5β-DHT, and m/z 664 for E2 (Haraguchi et al., 2012a). Unlike HPLC analysis, GC–MS analysis was capable of separating several pairs of isomers: 7α- and 7β-OH PREG; 3α,5α- and 3β,5β-THP; and 5α- and 5β-DHT (Haraguchi et al., 2012a). As summarized in Fig. 3, the neurosteroids produced in the pineal gland were identified as 7α- and 7β-OH PREG, PROG, 3α,5α- and 3β,5β-THP, AD, T, 5α- and 5β-DHT, and E2 (Haraguchi et al., 2012a). In sum, molecular and biochemical techniques have demonstrated that the pineal gland produces a variety of neurosteroids from CHOL via PREG in the juvenile birds. This is the first observation of de novo neurosteroidogenesis in the pineal gland in any vertebrate.

To investigate major neurosteroids formed and released in the pineal gland, the pineal glands from quail chicks were cultured in medium 199 with 7H-PREG. HPLC analysis revealed that PREG was converted primarily into 7α- and/or 7β-OH PREG and 3α,5α- and/or 3β,5β-THP in the pineal gland (Haraguchi et al., 2012a). The formation of 7α- and/or 7β-OH PREG and the expression of P450sc5c mRNA in the pineal gland also occurred in adult quail, while they were significantly lower than those in juvenile quail (Haraguchi et al., 2012a). 3α,5α- and/or 3β,5β-THP synthesis and 5α-reductase mRNA expression in the pineal gland were also higher in juveniles than in adults (Haraguchi et al., 2012a). Surprisingly, in juvenile quail, the formation of 7α- and/or 7β-OH PREG and the expression of P450sc5c mRNA were higher in the pineal gland than those in the cerebellum and diencephalon (Haraguchi et al., 2012a). 3α,5α- and/or 3β,5β-THP synthesis and 5α-reductase mRNA expression were also higher in the pineal gland than those in the cerebellum and diencephalon in juvenile quail (Haraguchi et al., 2012a). It thus appears that the pineal gland produces 7α- and/or 7β-OH PREG and 3α,5α- and/or 3β,5β-THP far more abundantly than brain regions.

To clarify the release of neurosteroids from the pineal gland, the pineal glands of quail chicks were cultured in medium 199 and the released neurosteroids were measured by GC–MS. Significant amounts of 7α-OH PREG and 3α,5α-THP were found to be released from the pineal gland into the culture medium, unlike 7β-OH PREG and 3β,5β-THP (Haraguchi et al., 2012a). Accordingly, 7α-OH PREG and 3α,5α-THP are considered to be the major neurosteroids secreted from the pineal gland (Fig. 3; Haraguchi et al., 2012a).

3.3. Light-dependent synthesis of pineal 7α-OH PREG and its biological action on locomotor rhythms

The avian pineal gland is light-sensitive by several opsins including pinopin (Okano et al., 1994), red-sensitive cone pigment (Okano et al., 1997), melanopsin (Chaurasia et al., 2005) and neurexin (Yamashita et al., 2010). These opsins confer light-sensitivity on the pineal function governing rhythmic nighttime-specific production of melatonin (Fukada and Okano, 2002). Accordingly, the avian pineal gland has been widely used for studies on light-regulation of the circadian clock (Fukada and Okano, 2002). Analysis of light-regulated genes in the chicken pineal gland led to a new finding that the pineal gland actively produces 7α-OH PREG (Hatori et al., 2011).

The circadian clock controls daily rhythms of physiology and behavior. The phase of the circadian clock is adjusted by the changes of environmental conditions, such as light, temperature, and food, in a time-of-day-dependent manner (Hirota and Fukada, 2004). For example, a light pulse given at early night and late night induces phase delay and advance, respectively. Despite the importance of phase-dependent light response, its molecular mechanism still remains unclear. The chick pineal gland has been considered as a model to work on this issue (Fukada and Okano, 2002). The circadian clock genes, such as Per, Cry, Clock, and Bmal, are highly conserved between the chick and mouse. The transcription- and translation-based feedback loop of the clock genes forms a core part of the circadian clock. For example, the expression of AANAT gene encoding the rate limiting enzyme of melatonin synthesis is
Fig. 3. Identification of neurosteroids and biosynthetic pathways for neurosteroids in the pineal gland. (A) Pineal glands from male quail chicks were incubated with $^3$H-PREG and the extracts were subjected to HPLC analysis. The arrows indicate the elution positions of $^3$H-PREG (open arrow) and its metabolites (black arrows). (B) Comparisons of 3α,5α-THP synthesis in the pineal gland of adults and chicks of both sexes. Each column and vertical line represent the mean ± SEM ($n = 8$). **$P < 0.01$ vs. adults. Comparisons of 3α,5α-THP synthesis in the pineal gland, cerebellum and diencephalon of quail chicks of both sexes. Each column and vertical line represent the mean ± SEM ($n = 8$). **$P < 0.01$ vs. cerebellum or diencephalon. (C) Comparisons of 7α-OH PREG synthesis in the pineal gland of adults and chicks of both sexes. Each column and vertical line represent the mean ± SEM ($n = 8$). **$P < 0.01$ vs. adults. Comparisons of 7α-OH PREG synthesis in the pineal gland, cerebellum and diencephalon of quail chicks of both sexes. Each column and vertical line represent the mean ± SEM ($n = 8$). ** $P < 0.01$ vs. cerebellum or diencephalon. (D) Comparisons of 7α-OH PREG release from the pineal gland, cerebellum and diencephalon of male quail chicks by GC-MS. Each column and vertical line represent the mean ± SEM ($n = 6$). ****$P < 0.001$ vs. cerebellum or diencephalon. (E) Biosynthetic pathways for neurosteroids in the pineal gland. The arrows indicate the biosynthetic pathways of neurosteroids identified in the pineal glands of male quail chicks. The pineal gland actively produces a variety of neurosteroids de novo from CHOL. 7α-OH PREG and 3α,5α-THP are major products secreted by the pineal gland. P450sc, cytochrome P450 side-chain cleavage enzyme (gene name Cyp11a); P450scc, cytochrome P450 7α-hydroxylase (gene name Cyp17); 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (gene name Hsd3b); 3α-HSD, 3α-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (gene name Hsd3a); P450c17, CYP17, cytochrome P450 17α-hydroxylase/c17,20-lyase (gene name Cyp17); 17α-HSD, 17β-hydroxysteroid dehydrogenase (gene name Hsd17b); P450arom, cytochrome P450 aromatase (gene name Cyp19). See Haraguchi et al. (2012a) and the text for details.
regulated by CLOCK–BMAL1 through an E box element in the promoter (Chong et al., 2000). Chicken pineal transcriptome analyses identified many rhythmically expressed genes involved in a variety of physiological processes including melatonin synthesis (Bailey et al., 2003; Karaganis et al., 2008). Among the clock-related genes, E4bp4 encodes a basic leucine zipper transcription factor that represses Per2 expression. E4bp4 expression is induced by a light pulse given at early night that causes the phase delay (Fig. 4; Doi et al., 2001, 2004). GeneChip analysis was conducted to search for genes induced by a light pulse at different time points of the day in the chicken pineal gland (Hatori et al., 2011). Dark-reared juvenile chicks were exposed to light given at three time points of the day; daytime, early night or late night. The transcriptome analysis revealed that Insig-1 and HMG-CoA synthase genes show a light response pattern similar to that of E4bp4 that is strongly induced by a light pulse given at early night (Hatori et al., 2011). Insig-1 and HMG-CoA synthase genes are involved in CHOL biosynthesis and the targets of a transcription factor SREBP (sterol regulatory element-binding protein). Interestingly, the light pulse given at early night stimulated the formation of the active form of SREBP transcription factor and induced a number of SREBP-target genes involved in CHOL biosynthesis. By transcription reporter assays, SREBP activated the transcription from the chicken E4bp4 promoter, which indicates a novel role of SREBP in the photic input pathway of the circadian clock activated at early night (Fig. 4). In contrast, the transcriptome analysis revealed an activation of the heat shock and endoplasmic reticulum stress response pathways at late night (Hatori et al., 2011).

The photic induction of SREBP-target genes involved in CHOL biosynthesis raised the possibility that the production of CHOL (and its derivatives) may be up-regulated by light in the pineal gland. The analysis of neurosteroidogenesis eventually revealed that the chick pineal gland actively produces and secretes 7α-OH PREG (Hatori et al., 2011) that enhances locomotor behavior (Fig. 4; Tsutsui et al., 2008). In accordance with the transcriptional changes in response to the light pulse, 7α-OH PREG production was stimulated at specific time of the day. It was activated by a light pulse given at early night, but not at late night and daytime (Hatori et al., 2011). Furthermore, locomotor behavior of dark-reared juvenile chicks was stimulated by light exposure more strongly at early night than at late night and daytime (Hatori et al., 2011). Interestingly, the light-dependent stimulation of locomotor behavior at early night is reduced by Px (Hatori et al., 2011). Taken together, the production of pineal 7α-OH PREG is stimulated by light depending on the time-of-day under the control of the circadian clock, and these properties may be essential for the regulation of locomotor behavior (Fig. 4). In sum, the pineal gland appears to regulate sleep/wake cycles through dual hormonal signals: rhythmic production of melatonin and light-dependent production of 7α-OH PREG.

### 3.4. Biological action and mode of action of pineal 3α,5α-THP on Purkinje cell survival

The two major pineal neurosteroids, 7α-OH PREG and 3α,5α-THP, are abundantly released from the pineal gland of juvenile birds (Haraguchi et al., 2012a). Therefore, not only pineal 7α-OH PREG but also pineal 3α,5α-THP may play important roles in the avian brain during development. In birds, the pineal gland is located near the cerebellum. The Purkinje cell is known to be a principal cerebellar neuron that integrates the process of memory and learning. It has been reported that in birds and mammals, Px induces cell loss in the brain including Purkinje cells during development (Kilic et al., 2002; Tunc et al., 2006). This observation indicates that certain component(s) in the pineal gland contributes to Purkinje cell survival during development. Tsutsui and colleagues therefore hypothesized that 3α,5α-THP and/or 7α-OH PREG secreted by the pineal gland may play a role in preventing the death of developing Purkinje cells. To test this hypothesis, Tsutsui and colleagues conducted a series of experiments in the male juvenile quail. Px decreased the concentration of 3α,5α-THP in the cerebellum and induced apoptosis of Purkinje cells, whereas administration of 3α,5α-THP to Px birds increased the 3α,5α-THP concentration in the cerebellum and prevented apoptosis of Purkinje cells (Haraguchi et al., 2012a). Tsutsui and colleagues further indicated that pineal 3α,5α-THP reaches Purkinje cells in the cerebellum by diffusion shown by injection of 3H-3α,5α-THP close to the pineal lumen (Fig. 5; Haraguchi et al., 2012a). Thus, 3α,5α-THP secreted by the pineal gland is considered to be a key factor for Purkinje cell survival during development (Fig. 5). In contrast to 3α,5α-THP, administration of 7α-OH PREG to Px birds did not increase Purkinje cell survival (Haraguchi et al., 2012a). Although 7α-OH PREG did not facilitate Purkinje cell survival, this neurosteroid is involved in the regulation of locomotor rhythms in birds (Hatori et al., 2011; Tsutsui et al., 2008) as mentioned above.

The induction of cell death of Purkinje cells in the cerebellum by Px suggests that certain other component(s) in the pineal gland may contribute to Purkinje cell survival during development. However, pineal melatonin did not facilitate Purkinje cell survival during development and did not affect the formation of...
3α,5α-THP in the cerebellum in quail chicks (Haraguchi et al., 2012a). It is therefore considered that 3α,5α-THP but not melatonin acts as an important component of the pineal gland for Purkinje cell survival during development. 3α,5α-THP produced in the pineal gland may reach the target site within the cerebellum by diffusion, because a significant amount of 3α,5α-THP was released from the cultured pineal gland of juvenile quail (Haraguchi et al., 2012a). However, we cannot exclude the possibility of an anatomical link (e.g., blood vessels) from the pineal gland to the cerebellum.

Finally, Tsutsui and colleagues investigated the mode of action of pineal 3α,5α-THP on Purkinje cell survival. Caspase-3, a crucial mediator of apoptosis, is known to play an important role in Purkinje cell death in vertebrates (Matsunaga et al., 2004b; O ł kowski et al., 2008; Puig and Ferrier, 2001). Interestingly, Px increased the number of Purkinje cells that expressed active caspase-3 in quail chicks and administration of 3α,5α-THP to Px birds decreased the number of Purkinje cells expressing active caspase-3 (Haraguchi et al., 2012a). Accordingly, the neuroprotective effect of pineal 3α,5α-THP on Purkinje cells is accompanied with the decrease in caspase-3 activity during development. Tsutsui and colleagues thus proposed that 3α,5α-THP exerts antiapoptotic effects in Purkinje cells by suppressing the activity of caspase-3 during development (Fig. 5).

It is unclear whether the action of pineal 3α,5α-THP on caspase-3 activity in the Purkinje cell is rapid (i.e., mediated through a membrane receptor) or slow (i.e., involving transcriptional activation). On the other hand, the action of 3α,5α-THP produced in the brain is likely mediated through interaction with the GABA_A receptor pathway, since 3α,5α-THP is a potent allosteric modulator of GABA_A receptor (Lambert et al., 1995; Paul and Purdy, 1992). However, the intracellular signaling pathway of pineal 3α,5α-THP suppressing the activity of caspase-3 in the Purkinje cell remains unclear. We need to clarify the intracellular signaling pathway exerting neuroprotective effect of pineal 3α,5α-THP in the Purkinje cell.

3.5. Summary

The pineal gland actively produces neurosteroids de novo from CHOL in juvenile birds. This is a new aspect of the biosynthesis of neurosteroids, because up until recently, we believed that neurosteroids are produced only in neurons and glial cells in the central and peripheral nervous systems. 7α-OH PREG is a major pineal neurosteroid that regulates locomotor rhythms of juvenile birds, connecting light-induced gene expression with locomotion. The other major pineal neurosteroid 3α,5α-THP prevents cell death of Purkinje cells by suppressing the activity of caspase-3 during development. These are new functions of the pineal gland by the actions of pineal neurosteroids. Interaction of brain and pineal neurosteroids in the regulation of brain development and functions deserve further investigations.

4. Conclusions

Identification of novel neurosteroids is essential for the progress of neuroendocrinology. We discovered 7α-OH PREG, a novel bioactive neurosteroid that acts as a key regulator for inducing locomotor behavior by means of the dopaminergic system. We further discovered that the pineal gland, an endocrine organ located close to the brain, is an important site of production of neurosteroids de novo from CHOL. The pineal gland secretes 7α-OH PREG and 3α,5α-THP that are involved in locomotor rhythms and neuronal survival, respectively. Thus, our studies over the past two decades have significantly broadened the horizons of this field of research by identifying novel neurosteroids in vertebrates that have opened new lines of scientific investigation in neuroendocrinology.

Disclosure statement

The authors have nothing to disclose.

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