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NEONATAL DEXAMETHASONE EXPOSURE DOWN-REGULATES GnRH EXPRESSION THROUGH THE GnIH PATHWAY IN FEMALE MICE

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Abstract—Synthetic glucocorticoid (dexamethasone; DEX) treatment during the neonatal stage is known to affect reproductive activity. However, it is still unknown whether neonatal stress activates gonadotropin-inhibitory hormone (GnIH) synthesizing cells in the dorsomedial hypothalamus (DMH), which could have pronounced suppressive action on gonadotropin-releasing hormone (GnRH) neurons, leading to delayed pubertal onset. This study was designed to determine the effect of neonatal DEX (1.0 mg/kg) exposure on reproductive maturation. Therefore, *GnRH*, *GnIH* and *GnIH* receptors, G-protein coupled receptors (*GPR*) 147 and *GPR*74 mRNA levels were measured using quantitative real-time PCR in female mice at postnatal (P) days 21, 30 and in estrus stage mice, aged between P45–50. DEX-treated females of P45–50 had delayed vaginal opening, and irregular estrus cycles and lower *GnRH* expression in the preoptic area (POA) when compared with age-matched controls. The expression levels of *GPR147* and *GPR74* mRNA in the POA increased significantly in DEX-treated female mice of P21 and P45–50 compared to controls. In addition, *GPR147* and *GPR74* mRNA expression was observed in laser captured single GnRH neurons in the POA. Although there was no difference in *GnIH* mRNA expression in the DMH, immunostained GnIH cell numbers in the DMH increased in DEX-treated females of P45–50 compared to controls. Taken together, the results show that the delayed pubertal onset could be due to the inhibition of *GnRH* gene expression after neonatal DEX treatment, which may be accounted for in part by the inhibitory signals from the up-regulated GnIH–GnIH receptor pathway to the POA.

Key words: glucocorticoid, GPR147, stress, reproduction, preoptic area, DMH.

INTRODUCTION

The activity of the hypothalamic–pituitary–gonadal (HPG) axis and pubertal onset are strictly regulated by gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (POA). Gonadotropin-inhibitory hormone (GnIH) expressed in the dorsomedial hypothalamus (DMH) has pronounced inhibitory action on GnRH neuronal activity (Ducret et al., 2009; Wu et al., 2009) and Luteinizing hormone (LH) release (Kriegsfeld et al., 2006; Murakami et al., 2008; Anderson et al., 2009; Rizwan et al., 2009). GnIH acts on high affinity G-protein coupled receptors (GPR) 147 (also designated as OT7T022 or NPFF1) and low affinity receptors GPR74 in mammals (Hinuma et al., 2000). 80% of GnRH neurons in the starling coexpress *GPR*147 mRNA (Ubuka et al., 2008). In rodents, GnIH immunoreactive fibers are seen in close apposition to GnRH neurons in the POA (Johnson et al., 2007; Kriegsfeld et al., 2010; Soga et al., 2010). These results suggest that GnIH can act directly on GnRH activity in the POA, although there are reports suggesting that GnIH exerts hypophysiotropic action at the level of the pituitary (Clarke et al., 2008; Murakami et al., 2008; Pineda et al., 2010). In fact, electrophysiological studies in mice have shown that GnIH directly inhibited firing of 45% of GnRH neuronal population (Ducret et al., 2009) possibly via a postsynaptic mechanism (Wu et al., 2009).

Glucocorticoids are essential hormonal factors in the hypothalamic–pituitary–adrenal (HPA) axis that maintain the body's homeostasis including reproduction and normal brain functions. Early life stress or exposure to exogenous glucocorticoids during developmental stages has long-lasting effects which include inhibition of somatic growth, cerebral atrophy, endocrine disruption and consequential increase in anxiety-related behaviors (Calogero et al., 1990; Anisman et al., 1998; Woods et al., 1999; He et al., 2004; Kamphuis et al., 2004). In particular, the expression and epigenetic modulation of neuron-specific glucocorticoid receptor (GR) gene in the forebrain and hippocampus are influenced by early life stress (Meaney et al., 1996; McGowan et al., 2009). Elevated glucocorticoids due to treatment or chronic stress, affect reproductive activity such as testosterone metabolism, sexual behavior and normal sexual differentiation in male and female animals (Henry et al., 1996; Rhees et al., 2001), which leads to sexual dysfunction (Ferin, 1999; Berga, 2008; Lupien et al., 2009). Additionally, stressful events in humans such as childhood trauma and abuse are

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Abbreviations: ANOVA, analysis of variance; Cyp, cyclophilin; cDMH, compact portion of DMH; DAB, 3,3′diaminobenzidine tetrahydrochloride; DEPC, diethylpyrocarbonate; DEX, dexamethasone; DMH, dorsomedial hypothalamus; DTM, dorsal tuberomamillary nucleus; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; GFAP, glial fibrillary acidic protein; GPR, G-protein coupled receptors; GR, glucocorticoid receptor; HPA, the hypothalamic-pituitary-adrenal; HPG, the hypothalamic-pituitary-gonadal; IgG, immunoglobulin G; LH, Luteinizing hormone; P, postnatal; PB, phosphate buffer; PBS, phosphate buffered saline; PFA, paraformaldehyde; POA, preoptic area; rpm, revolutions per minute; vDMH, ventral portion of DMH.

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known to increase susceptibility to reproductive illness and mental disorder during adulthood perhaps through reprograming of the HPG axis by glucocorticoids in humans (Heim and Nemeroff, 2001).

Synthetic glucocorticoid (dexamethasone; DEX) is commonly used to promote fetal lung development in cases of chronic lung disease in newborn infants (Halliday et al., 2003, 2009, 2010). Potential side effects of postnatal DEX on neurodevelopment have been studied in rats (Camm et al., 2011). In fact, DEX treatment in humans is known to increase the incidence of cerebral palsy (Shinwell et al., 2000; Powell et al., 2006). However, there are relatively no clinical reports of delayed puberty by neonatal DEX treatment. Hence, it is important to examine the impact of neonatal DEX treatment on reproductive maturation, leading to delayed puberty and impaired sexual behavior in rodents (Smith and Waddell, 2000; Iwasa et al., 2011a).

It is well known that stress-induced glucocorticoids inhibit GnRH neuronal activity and LH release (DeFranco et al., 1994; Tilbrook et al., 2002; Gore et al., 2006) through GRs expressed in a small percent of GnRH neurons (Dondi et al., 2005). Moreover, both acute and chronic stress-induced glucocorticoids cause an increase in *GnIH* gene expression and *cfos* expression in GnIH neurons (Kirby et al., 2009; Kaewwongse et al., 2011), thereby, inhibiting reproductive functions. In rats, 53% of GnIH neurons express GRs and adrenalectomy blocks stress-induced *GnIH* gene expression (Kirby et al., 2009), indicating a direct effect of glucocorticoids on the function of GnIH neurons.

In this study, we hypothesize that exposure to DEX during early life causes abnormal development of GnIH–GnRH pathway in the brain, which results in altered reproductive activity. However, the mechanism underlying the activation of GnIH–GnIH receptors signaling following neonatal glucocorticoid exposure is not well understood. Therefore, this study examined the effect of neonatal DEX exposure on *GnRH*, *GnIH* receptors (*GPR*147 and *GPR*74) expression pattern in the POA and GnIH expression in the DMH, during pre-pubertal and pubertal stage of female mice.

EXPERIMENTAL PROCEDURES

Animals

Experiments were carried out using C57BL/6J female mice (CLEA Japan, Inc) in accordance with the guidelines for the care and use of animals by Monash University Sunway Campus, Malaysia (SOBSB/MY/2010/47). These animals were housed in groups of three or four under a 12: 12 h light: dark schedule (lights on 12:00 h) at 25 °C. Food and water were available *ad libitum*.

Treatment

Gestational-timed pregnant mice were housed in individual cages. The pups per litter were culled 6–8 to receive the same treatment. A balanced male to female ratio was maintained to minimize the effect of litter size on nutrition, body weight and maternal care. For studies of the effects of neonatal glucocorticoids treatment, pups were given daily subcutaneous injections of DEX 21-phosphate disodium salt (Sigma, Chemical Co., St. Louis, MO, USA) at the dose of 1.0 mg/kg on neonatal days 1 through 4 whereas controls received equivalent volumes (1 μl/g) of saline vehicle. All pups were separated from the mother on day 21 after birth and group-housed with littermates per treatment (Fig. 1A) and monitored for the day of vaginal opening and followed by daily estrus cycle check by vaginal smear analysis. All pups from a litter were treated the same way (i.e. type of treatment and sampling time point). For each sampling time point, 2–3 different litters were treated to get sufficient number of female pups for biochemical experiments. DEX or saline treated pups from all litters were arbitrarily divided into three groups for different sampling time points on postnatal day (P) 21(control = 11, DEX = 7), P30 (control = 7, DEX = 6) and estrus mice, aged between P 45–50 (control = 15, DEX = 15). In addition, the P45–50 group was further divided into two groups for gene expression study (control = 8, DEX = 6) and immunohistochemical study (control = 7, DEX = 9) (Fig. 1A).

All female mice at each sampling point were deeply anesthetized using sodium pentobarbital solution (100 mg/kg body weight, Ceva Sante Animale, Libourne, France) and the brains were collected and kept at −80 °C until use for gene expression study.

Gene expression study

Frozen coronal sections (60 μm thick) of the brain were prepared from the beginning of the lateral veins to the end of the 3rd ventricle covering the POA and DMH (POA: bregma, 0.62–0.38 mm, DMH: bregma, −1.46 mm to −2.18 mm) in a cryostat. The POA and DMH regions were dissected out from the brain sections using a sharp blade (set at same temperature as the cryostat) and immediately transferred to 200 μl of TRIZol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted following the manufacturers' protocol. In brief, the brain tissue was homogenized in 200 μl of TRIZol reagent, mixed with 40 μl of chloroform and centrifuged at 12,000 rpm (revolutions per minute) for 15 min at 4 °C. The RNA containing aqueous layer was purified by ethanol precipitation and the total RNA pellet was dissolved in 25 μl of diethylpyrocarbonate (DEPC)-treated H_2O.

Total RNA (1 μg) was reverse transcribed using ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol in a final volume of 20 μl. The cDNA was subjected to real-time PCR for *GnRH*, *GPR147*, *GPR74*, and *GnIH* mRNAs using an ABI 7500 Real-Time PCR system (Applied Biosystems) and Sequence Detection Software ver 1.3.1 (Applied Biosystems). Relative expression values of the above genes were obtained by normalizing threshold cycle (Ct) values of genes of interest against Ct value of cyclophilin (*Cyp*) ($2^{\wedge}[Ct_{Cyp} - Ct_{Gene\ of\ interest}]$).

The PCR mixture contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μM each forward and reverse primers, and 1 μl of sample cDNA. PCR mixture was heated at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min followed by melting curve analysis. The nucleotide sequences of the primers used in this study are presented in Table 1. To confirm the PCR specificity, the PCR products were sequenced using Big Dye, ABI 3130 Genetic Analyzer and Sequence Analysis Software (Applied Biosystems).

Immunohistochemistry

Female adult mice (n = 5, 9 weeks old) were anaesthetized with sodium pentobarbital (100 mg/kg body weight) and perfused through the heart with 20 ml of 0.1-M phosphate-buffered saline (PBS) heparinized at 1:100 (heparin; Wako Pure Chemical Industries, Ltd., Osaka, Japan) followed by 35 ml of cold 4% paraformaldehyde (PFA) (buffered in 0.1 M phosphate buffer [PB]). For single-cell laser capture purposes, all solutions were treated with DEPC. The brains were dissected and postfixed in 4% PFA for