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Molecules and Cells

Regulation of the Female Rat Estrous Cycle by a Neural Cell-Specific Epidermal Growth Factor-like Repeat Domain Containing Protein, NELL2

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NELL2, a protein containing epidermal growth factor-like repeat domains, is predominantly expressed in the nervous system. In the mammalian brain, NELL2 expression is mostly neuronal. Previously we found that NELL2 is involved in the onset of female puberty by regulating the release of gonadotropin-releasing hormone (GnRH), and in normal male sexual behavior by controlling the development of the sexually dimorphic nucleus of the preoptic area (POA). In this study we investigated the effect of NELL2 on the female rat estrous cycle. NELL2 expression in the POA was highest during the proestrous phase. NELL2 mRNA levels in the POA were increased by estrogen treatment in ovariectomized female rats. Blocking NELL2 synthesis in the female rat hypothalamus decreased the expression of kisspeptin 1, an important regulator of the GnRH neuronal apparatus, and resulted in disruption of the estrous cycle at the diestrous phase. These results indicate that NELL2 is involved in the maintenance of the normal female reproductive cycle in mammals.

INTRODUCTION

A protein that is strongly expressed in neural tissues and containing EGF-like domains (Nel) was first identified in the chick embryo (Matsuhashi et al., 1995). Subsequently two Nel-like proteins, NELL1 and NELL2, were isolated from a human fetal brain library (Watanabe et al., 1996). Of these two mammalian NEL proteins, NELL2 appears to be the mammalian counterpart to chick Nel (Kuroda et al., 1999; Oyasu et al., 2000), because, like Nel, NELL2 is almost exclusively expressed in neurons (Oyasu et al., 2000).

Because NELL2 is ubiquitously expressed in neurons, it was considered to play general roles in maintaining neuronal functions (Kim et al., 2002; Oyasu et al., 2000). However, we recently found that NELL2 is specifically expressed in glutamatergic neurons, but not in the gonadotropin-releasing hormone (GnRH) neurons of the rat hypothalamus (Ha et al., 2008). *In vivo* blockade of NELL2 synthesis results in decreased GnRH release (Ha et al., 2008), suggesting that NELL2 is involved in the GnRH release apparatus via regulating glutamate or other neuronal networks that control GnRH release. The transsynaptic networks controlling GnRH secretion are many (review, Levine et al., 1991) and subject to the modulatory influence of gonadal steroids such as estradiol (E2) (review, Horvath et al., 1997). The most important excitatory components of this transsynaptic system are provided by glutamatergic neurons and kisspeptin 1 (KiSS1)-producing neurons (review, Seminara, 2005).

In an earlier study, we showed that expression of NELL2 is regulated by E2 in the rat hypothalamus (Choi et al., 2001). Promoter assays using the 5′ flanking region of the NELL2 gene further revealed that E2 and its receptors (ERα and ERβ) directly regulate transcription of the NELL2 gene (Choi et al., 2010). We also identified that NELL2 is specifically expressed in hypothalamic cells expressing ERα and is involved in E2-dependent hypothalamic sexual differentiation (Jeong et al., 2008a) and reproductive maturation (Ha et al., 2008), while GnRH neurons do not express ERα, a major feedback regulator for the GnRH neural apparatus (Herbison and Theodosis, 1992). Therefore, NELL2 might be essential to the E2-dependent control of neuroendocrine events related to reproduction, such as puberty initiation (Ha et al., 2008) and female reproductive cyclicity.

In this study, we investigated the role of NELL2 in the control of the female rat estrous cycle. An antisense (AS) oligodeoxynucleotide (ODN) against the translation initiation site of NELL2 mRNA was injected into the lateral ventricle of adult female rat brains. This injection disrupted NELL2 synthesis, and in turn, resulted in a decrease of KiSS1 mRNA from the hypothalamic preoptic area (POA). Moreover, the estrous cycle of female rats ceased at the diestrous phase after AS ODN injection. These results suggest that NELL2 is a component of the machinery regulating GnRH neurons, and thereby, facilitates the maintenance of the normal reproductive cycle in mammals.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats (8 weeks old, body weight 190-210 g) were purchased from Daehan Animal Breeding Company (Korea) and cared for in accordance with the Univer-

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sity of Ulsan regulations for the care and use of experimental animals. Animals were housed under controlled temperature (23-25°C) and light (12 h-light/dark cycle, lights on at 07:00 h) conditions. Food and water were provided ad libitum. The estrous cycle was monitored via vaginal smears and rats showing at least three consecutive 4-day cycles were used. Some animals were ovariectomized (OVX) under anesthesia (2.5% tribromoethanol; Sigma, USA), and subcutaneously (sc) injected with estradiol benzoate (50 μg/kg in sesame oil carrier, Sigma) one week after OVX. Two days later the animals were humanely sacrificed by decapitation and their brains were quickly removed. The POA fragments were collected, frozen on dry ice, and stored at -80°C until use.

Real-time PCR

Total RNA (2 μg) was reverse-transcribed and amplified by real-time PCR using the following primer sets: NELL2 (NCBI GenBank Database No., AY089719) sense primer, 5′-CAGGG GACTG CGTGC ACGAC-3′; antisense primer, 5′-GCACT GTGGG TCCAC TGAGC A-3′; KiSS1 (NCBI GenBank Database No., AY196983.1) sense primer, 5′-TGGCA CCTGT GGTGA ACCCT GAAC-3′; antisense primer, 5′-ATCAG GCGAC TGCGG GTGGC ACAC-3′. Real-time PCR reactions [20 μl total volume, containing 5 pmol primer, 10 μl SYBR green dye (Quiagen, USA) and 2 μl cDNA] were performed using the DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research Inc., USA) for approximately 40 cycles.

Western blotting

The harvested POA tissues were homogenized in T-PER lysis buffer (Pierce Chemical Co., USA) with a protease inhibitor cocktail (3 mM aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml leupeptin) and 1 mM sodium orthovanadate, pH 6.8. The extracted proteins (20 μg) were resuspended in sample buffer containing 62 mM Tris-HCl, pH 6.8, 1 mM EDTA, 10% glycerol, 5% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and separated by SDS-polyacrylamide gel electrophoresis. Proteins on the gel were transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) by an electrophoretic transfer cell (Bio-Rad Laboratories, USA). The membrane was incubated with primary antibody (rabbit anti-NELL2, 1:1000) (Ha et al., 2008) for 1 h in hybridization buffer (1x Tris-buffered saline with 0.1% Tween 20, 1% milk) and followed by incubation with secondary antibody (anti-rabbit IgG, 1:2000; Amersham Biosciences). The immunoreactions were detected with an enhanced chemiluminescence detection kit (Amersham Biosciences) according to the supplied protocol.

Intracerebroventricular (icv) administration of AS NELL2 ODN

To block NELL2 synthesis in the rat brain, a phosphorothioate AS NELL2 ODN (GenoTech Corp., Korea) was icv delivered to the brain. The AS NELL2 ODN, used to disrupt NELL2 synthesis (5′-CCG GGA TTC CAT GGC GTG CAT-3′), was directed against the sequence surrounding the ATG codon of NELL2 mRNA as previously reported (Ha et al., 2008; Jeong et al., 2008a). As a control, a scrambled (SCR) sequence of identical base composition was used (5′-TAT CGC ATG CGG GCC TAT GCG-3′). For the icv injection, the ODNs were diluted to a final concentration of 100 ng/ml in artificial cerebrospinal fluid (ACSF) (Kim et al., 2002). Immediately after OVX surgery, a polyethylene cannula (O.D. 1.05 mm, I.D. 0.35 mm) was stereotaxically implanted into the lateral ventricle (coordinates: 1.0 mm caudal to the bregma; 4.0 mm vertical from the dura mater; 1.6 mm lateral from the midsagittal line). After 1 week of recovery, ODNs (2 nmol in 4 μl ACSF) were injected with a Hamilton syringe once per day for 2 consecutive days. At the first ODN injection, E2 was also injected sc. All animals were sacrificed 24 h after the second ODN administration and their POA tissues were collected.

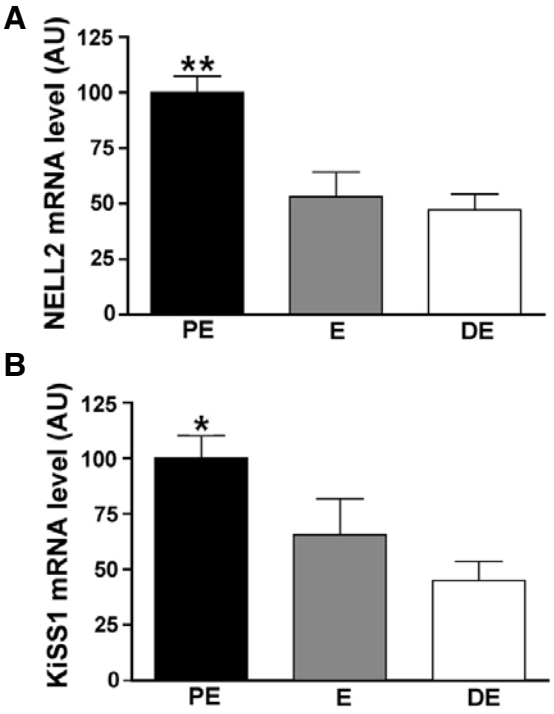

Fig. 1. Changes in NELL2 and KiSS1 mRNA levels in the POA during the female rat estrous cycle. Real-time PCR analysis revealed changes in NELL2 (A) and KiSS1 (B) mRNA levels in the POA samples obtained at proestrous (PE), estrous (E), and diestrous (DE) phases of the estrous cycle. Results are expressed as the mean ± S.E.M. of four independent experiments. **$p < 0.01$, *$p < 0.05$.

Statistical analysis

Differences among groups were analyzed using a one-way ANOVA with Dunnett's multiple comparisons test. Student's *t*-test was used for comparisons between two groups.

RESULTS

Changes in NELL2 and KiSS1 mRNA levels in the rat POA during estrous cycle

Stages of the estrous cycle were determined based on the collected vaginal smears and classified as proestrous, estrous and diestrous phases. Expression of NELL2 and KiSS1 mRNA was detected in the adult female rat POA by means of real time-PCR during the normal estrous cycle. NELL2 and KiSS1 mRNA levels significantly changed during the estrous cycle (Fig. 1). Notably, both mRNA species were most highly expressed in the proestrous phase, suggesting that these proteins are involved in the control of the female estrous cycle.

Change of NELL2 protein and KiSS1 mRNA levels by E2

Because E2 is one of the most important peripheral regulators of the female estrous cycle, we sought to determine if an increase in NELL2 and KiSS1 expression at proestrous phase

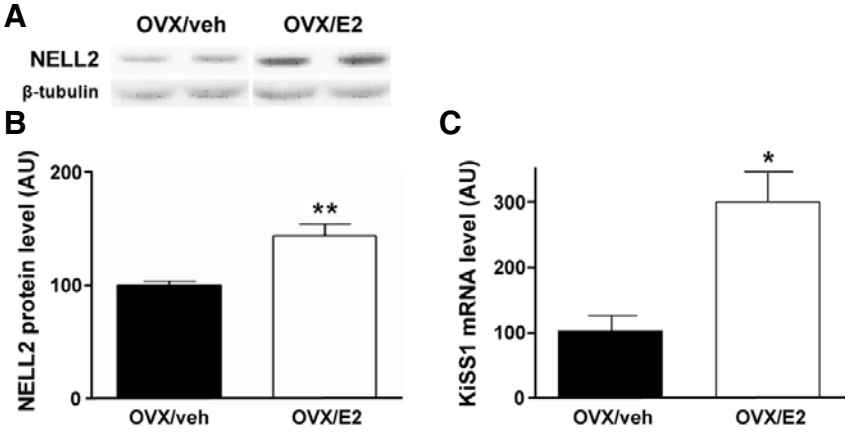

Fig. 2. Changes in NELL2 protein and KiSS1 mRNA levels in the POA by estradiol benzoate (E2) treatment. The POA tissues were collected from ovariectomized (OVX) rat brain 2 days after sc injection with E2 (50 μg/kg) or sesame oil (veh). (A) Representative Western blots showing the separated protein bands of NELL2 and the internal control, β-tubulin. (B) Calculated change in NELL2 protein levels (n = 4) showing the effect of sc injection with E2. (C) Real-time PCR analysis (n = 4) revealed changes in KiSS1 mRNA levels by E2 treatment. *p < 0.05. **p < 0.01 vs. Veh.

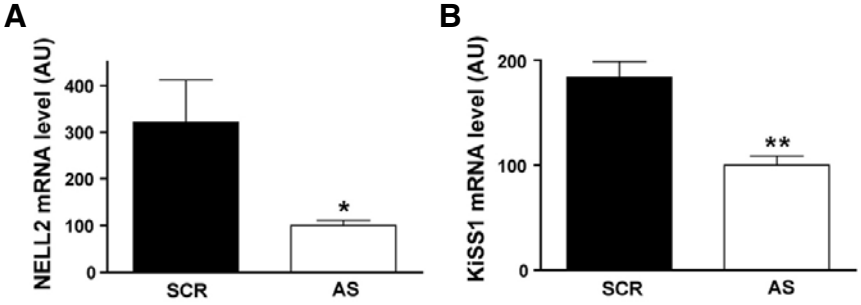

Fig. 3. Effects of blocking NELL2 synthesis on the change in KiSS1 mRNA level in the POA. POA samples were derived from OVX and E2 injected female rats after daily icv injection of the AS NELL2 ODN for 2 days. Real-time PCR analysis (n = 5) revealed the effect of the AS ODN on the NELL2 (A) and KiSS1 (B) mRNA levels. *p < 0.05, **p < 0.01 vs. SCR.

was due to a feedback effect of E2 at this phase. Western blot analysis revealed that NELL2 expression was significantly increased by E2 in the POA of OVX female rats (Figs. 2A and 2B), which concurs with our previous finding of positive E2 action on NELL2 expression (Choi et al., 2010). Real-time PCR analysis also revealed that E2 increased KiSS1 mRNA levels in the POA (Fig. 2C).

Effect of NELL2 synthesis blockade on the change in KiSS1 mRNA levels

KiSS1 has emerged as the most important regulator of the GnRH neuronal apparatus under the influence of circulating E2 (Navarro et al., 2011), and changes in expression of KiSS1 and NELL2 follow similar patterns during the estrous cycle. Thus, to determine the effect of blocking NELL2 synthesis on the KiSS1 mRNA expression, the AS NELL2 ODN was injected icv into the brains of OVX female rats receiving E2. KiSS1 mRNA levels in the POA were analyzed by real-time PCR. The AS ODN effectively decreased NELL2 mRNA (Fig. 3A) as previously reported (Ha et al., 2008; Jeong et al., 2008a), and moreover, resulted in a decrease of KiSS1 mRNA levels in the POA tissues (Fig. 3B), suggesting that NELL2 is involved in the regulation of KiSS1 expression.

Effect of NELL2 synthesis blockade on the reproductive cycle of female rats

To determine the physiological importance of NELL2 function in the control of the female reproductive cycle, adult female rats with regular estrous cycles were injected icv with AS ODN daily for one week. Examination of the estrous cycle subsequent to initiation of treatment showed disruption of estrous cyclicity within a few days of injection (Fig. 4). AS ODN injected rats showed a preponderance of days in the diestrous phase of the cycle and a concomitant reduction of days in the proestrous and estrous phases. In contrast, control animals injected with ACSF and SCR ODN showed a normal estrous cyclicity (Fig. 4A). After 10-15 days, animals that stopped receiving AS NELL2 ODN injections recovered normal cyclicity. Animals that were injected with AS NELL2 ODN stayed at a significantly greater percentage of diestrous days and exhibited significantly fewer days at estrous phase (Fig. 4B).

DISCUSSION

These results suggest that NELL2 acts as a component of the machinery regulating the normal reproductive cycle in female rats, probably through control of KiSS1 gene expression. NELL2 expression varied with the female rat estrous cycle, reaching peak levels at proestrous phase. We also showed that an AS ODN-induced decrease of NELL2 synthesis in the POA caused cessation of the estrous cycle at the diestrous phase. Moreover, decreased synthesis of NELL2 resulted in a decreased level of KiSS1 mRNA in the POA.

The results of our previous reports suggested that NELL2 is important in the regulation of E2-dependent differentiation of the hypothalamus (Choi et al., 2001), and plays an important role in regulation of GnRH release by acting on glutamatergic neurons (Ha et al., 2008). Moreover, it is involved in the development of the sexually dimorphic nucleus of the male rat POA (Jeong et al., 2008a) by mediating the survival-promoting effect of E2 on neuronal cells (Choi et al., 2010). This suggests that NELL2 is involved in maintaining the normalcy of male sexual behavior. In this study we demonstrated that NELL2 is also important for maintenance of the female reproductive cycle.

We found that the expression of NELL2 mRNA peaked at the proestrous phase, when circulating E2 concentrations are high-

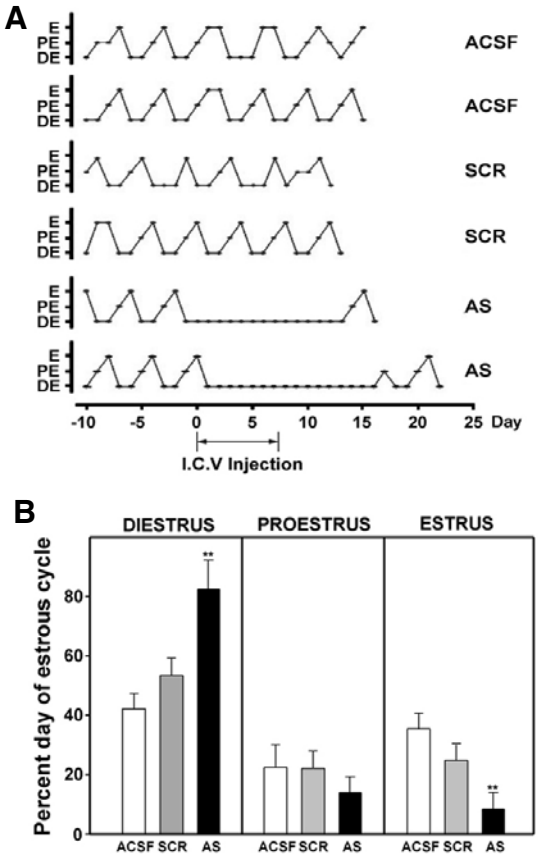

Fig. 4. Effects of AS NELL2 ODN on female rat reproductive cyclicity. Vaginal smears were performed to determine the estrous cycle of female rats that received icv administration of either the AS NELL2 ODN, or artificial cerebrospinal fluid (ACSF) and scrambled (SCR) ODN. (A) Representative examples showing disruption of the estrous cycle by the AS ODN, compared to normal cyclicity of ACSF and SCR injected control animals. Icv administration was applied for one week (indicated as arrow from day 0). DE, diestrus; E, estrus; PE, proestrus. (B) Mean percentage of time in each estrous cycle phase calculated from 6 individual animals for each experimental group. **, $p < 0.01$ vs. ACSF and SCR.

est (Saito et al., 2009). This finding is consistent with the results of our previous studies showing that E2 activates NELL2 transcription (Choi et al., 2010) and that NELL2 is expressed in the ERα expressing hypothalamic cells (Choi et al., 2010; Jeong et al., 2008a).

The estrous cycle is characterized by reproductive receptivity, changes in vaginal and uterine wall morphology, and fluctuation of serum E2 concentrations (Wood et al., 2007). E2 plays a pivotal role in the estrous cycle by regulating the expression of GnRH in the hypothalamus (Review, Smith and Jennes, 2001). The female reproductive cycle is regulated by a sequence of neuroendocrine events: hypothalamic GnRH release and subsequent release of gonadotropins, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), from the anterior pituitary regulates the ovaries; E2 secreted from the ovaries exerts a feedback effect on the GnRH and gonadotropin release (Review, Horvath et al., 1997). In rodents, the POA is the center of GnRH synthesis and release in response to E2 (Williams et al., 2011). However, GnRH-releasing neurons express neither ERα (Wintermantel et al., 2006), nor progesterone receptor (Skinner et al., 2001). GnRH-releasing neurons express ERβ (Hrabovszky et al., 2001), but this receptor is probably not functionally important to the central control of the reproductive axis (Dorling et al., 2003). Therefore, the feedback action of E2 on GnRH-releasing neurons may be conveyed by ERα-expressing neurons (Dorling et al., 2003). Our studies revealed that one such candidate neuron is the NELL2 expressing neuron (Choi et al., 2010; Ha et al., 2008).

KiSS1, also known as metastatin, was first isolated from human placenta and proposed to be the natural ligand for a G protein-coupled receptor, GPR54 (Ohtaki et al., 2001). Peripheral (Matsui et al., 2004) or icv (Navarro et al., 2004) injection of KiSS1 induced profound stimulation of LH secretion in prepubertal rats, and a genetic alteration leading to homozygous loss of function of GPR54 impaired pubertal development in mice (Seminara et al., 2003). In the rodent POA, E2 induced KiSS1 expression, implying that these KiSS1-secreting neurons play a role in the circulating E2 feedback regulation of GnRH secretion (Navarro et al., 2009). A number of studies have demonstrated that the KiSS1 system is a major gatekeeper of reproductive function in mammals (review, Seminara, 2005). The hypothalamic KiSS1 system has been proposed as an essential factor integrating a variety of regulatory inputs (such as sex steroids and energy status) into the regulation of GnRH-releasing neurons (Navarro et al., 2009).

In the present study, blocking NELL2 synthesis induced a decrease of KiSS1 expression in the POA and resulted in cessation of the female estrous cycle. Thus, KiSS1 activity may be a possible pathway through which NELL2 acts to regulate the GnRH neuronal apparatus. Though we did not demonstrate the detailed anatomical relationship between NELL2 and KiSS1 in this study, previous studies (including ours) revealed that NELL2 and KiSS1 are expressed in the same areas of the POA (Jeong et al., 2008b; Smith et al., 2005). Our previous studies revealed that NELL2 is specifically expressed in the glutamatergic but not in GABAergic neurons in the rat forebrain regions including cerebral cortex, hippocampus and hypothalamus, and is involved in the glutamate release (Ha et al., 2008). Therefore, NELL2-expressing neurons may affect KiSS1 expression via glutamate release, and in turn, regulate GnRH neurons, though this possibility certainly requires further studies.

In summary, we report that NELL2 may be an important modulator of E2 action in the regulation of reproductive cyclicity, possibly through the modulation of KiSS1 and the GnRH neuronal apparatus in the adult female rat POA.

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