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Neural Tissue-Specific Epidermal Growth Factor (EGF)-Like Domain Containing Protein, NELL2, Plays an Important Role in the Central Regulation of Puberty Onset in the Female Rat Hypothalamus

Chang Man Ha, Hae Mook Kang[1], and Byung Ju Lee*

Department of Biological Sciences, University of Ulsan, Ulsan 680-749;
[1]Department of Genetic Engineering, Chongju University, Chongju 360-764, Korea

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In the present study we determined if NELL2, a neural tissue-specific protein containing 6 epidermal growth factor (EGF)-like repeat domains, plays an important role in the regulation of puberty initiation in the rat hypothalamus. We originally found that NELL2 is a new estrogen-responsive gene in hypothalami derived from estrogen-sterilized and control rats using a PCR differential display. In the 40-day-old female rat hypothalamus, NELL2 was up-regulated by neonatal estrogen treatment. In situ hybridization histochemistry showed that NELL2 is very abundant in the ventromedial hypothalamic nucleus that is responsible for the control of sex behavior. NELL2 mRNA level in the medial basal hypothalamus showed a dramatic increase before female puberty onset, which suggests that NELL2 may be involved in the process regulating female puberty onset. We attemped to block NELL2 synthesis with intracerebroventricular injection of an antisense oligodeoxynucleotide (ODN) to the NELL2 mRNA, and examined its effect on the puberty onset of the female rat. The antisense ODN significantly delayed puberty initiation determined by vaginal opening. In summary, NELL2 may play an important role in the regulation of female puberty onset.

Sex differentiation of the female hypothalamus is dependent upon gonadal steroid environment during late gestational and early postnatal development (reviewed by Gorski, 1985). Exposure of neonatal female rats with a high dose of estrogen (E) or testosterone (T) determines steroid-dependent sex differentiation in the female brain (Barraclough, 1961; Gorski, 1963; Hayashi and Aihara, 1989; Hayashi et al., 1991), which results in permanent sterility during adulthood. In these E-sterilized rats (ESRs), puberty disappears and luteinizing hormone-releasing hormone (LHRH) and luteinizing hormone (LH) surges disappear by destruction of hypothalamo-pituitary-gonadal axis (Hayashi and Aihara, 1989; Faber et al., 1991; 1993; Pinilla et al., 1993). These data suggest that neonatal treatment of E may cause changes in the organizational pattern of brain areas responsible for the sexual differentiation preceding normal puberty initiation. How gonadal steroids elicit these irreversible imprinting and what kinds of molecular mechanisms are involved are poorly understood. Using ESR model, we tried to identify genes involved in the hypothalamic sex differentiation of the female rat by polymerase chain reaction differential display (ddPCR). More than 30 gene transcriptions were differentially imprinted between hypothalami of 60-day old ESR and control females (Choi and Lee, 1999). One of the genes that we have identified by ddPCR is a rat neural tissue- specific protein containing 6 repeated epidermal growth factor (EGF)-like domains, NELL2 gene (Kuroda et al., 1999).

A neural tissue-specific protein containing EGF-like domains (NEL) was first identified from a chick embryo (Matusuhashi et al., 1995). Its expression was reported to be restricted only in the neural tissues after hatching, while its mRNA is found in all tissues during fetal development. Nel-like (NELL) genes were also identified from a human fetal brain library and were named as NELL1 and NELL2 (Watanabe et al., 1996). NELL2 is more closely related to chicken nel than NELL1 and shows brain-specific expression in the adult human (Watanabe et al., 1996). Recently, NELL2 was identified as a protein kinase C (PKC) binding protein, and was known to be phosphorylated by PKC (Kuroda et al., 1999; Kuroda and Tanizawa, 1999).

Although the molecular structure of NELL proteins has been well characterized, their functions in the brain are unclear. In the present study, we report that

* To whom correspondence should be addressed.
Tel: 82-52-259-2351, Fax: 82-52-259-1694
E-mail: bjlee@uou.ulsan.ac.kr

NELL2 is involved in the regulation of puberty onset of the female rat. We found that NELL2 is up-regulated in the E-sterilized adult female rat hypothalamus using PCR differential display (ddPCR). Steroid hormone-dependent expression of NELL2 gene was further confirmed by other approaches such as northern blot hybridization, and a possible role of NELL2 in the regulation of female puberty onset was investigated using an antisense oligodeoxynucleotide.

Materials and Methods

Cloning of NELL2 cDNA fragment

Partial cDNA of the rat NELL2 was cloned after reverse transcription-PCR using a primer set based on the known sequence (Kuroda et al., 1999). This PCR-cloned partial cDNA fragment was used as a cDNA probe for Northern blot hybridization and a template for the generation of cRNA probe for in situ hybridization histochemistry. For PCR amplification of NELL2 cDNA, the upstream primer (5'-CTA GAG CTG AAC AAC GAA TGA-3') and down stream primer (5'-TCA TGG TCT GAT CCT TGC ATT-3') were designed. The amplified product was inserted into pGEM-T easy vector (Promega) and sequenced using automatic DNA sequencer.

Animals, tissue and RNA preparation

Pregnant female rats of Sprague Dawley strain were maintained ad libitum under the condition of 14-h light and 10-h dark photocycle. Neonatal pubs were kept with their mother and s.c. injected daily with 100 μg of 17 β-estradiol-benzoate (EB) for 7 days. These ESRs and normal control rats were sacrificed at 0-, 6-, 28-, 31-, and 40 day of age and total hypothalamic tissues including preoptic area (POA) and medial basal hypothalamus (MBH) were dissected, snap-frozen in liquid nitrogen, and kept at -80℃ until RNA isolation. Experiments with animals were conducted in accordance with the University of Ulsan Regulations, which adopt the NIH Guide for the Care and Use of Laboratory Animals. RNA extraction was basically performed with acid phenol-chloroform method (Chomczynski and Sacchi, 1987).

In situ hybridization histochemistry

The procedure employed is that described previously (Simmons et al., 1989) with minor modifications. The brains were fixed by transcardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), followed by overnight postfixation in the same fixative, containing 10% sucrose. After blocking regions of interest, tissues were frozen on dry ice and stored at -80℃ until sectioning. Twenty micrometer sections were obtained with a sliding microtome, adhered to ProbeOn^{TM} Plus microscope slides (Fisher Scientific), and dried under a vacuum overnight before hybridization. Sections were overlaid with 70 μl of hybridization solution containing 5×10^6 cpm of NELL2 cRNA probe per ml and hybridized for overnight at 60℃. Antisense RNA probe for in situ hybridization was transcribed from the linearized template with RNA polymerase in the presence of ^{35}S-UTP (Amersham), and separated from free isotope with a Nick column (Pharmacia). Posthybridization washes were carried out as recommended (Simmons et al., 1989). Some sections were dipped in NTB_2 emulsion and developed after 3 wk exposure.

Phases of postnatal development

Change in hypothalamic NELL2 mRNA levels was determined at 0-, 6-, 28-, 31-, and 40-day of age in ESR and control animals. Animals used for the determination of hypothalamic mRNA levels during the pubertal process were classified according to the established criteria (Ojeda and Urbanski, 1994). According to these criteria, the juvenile period in the female rat extends from postnatal days 21-30. Thus the 28-day-old animals used in this study can be considered as late juvenile and termed as anestrus (AE). Their vagina had no sign of activation and the uterine weighed 60 mg or less, without accumulation of intrauterine fluid. Animals with enlarged uteri and detectable intrauterine fluid were assigned to the early proestrous (EP) phase, which precedes the day of the first preovulatory surge of gonadotropins. Animals with the uterine weight of at least 200 mg and the uterus ballooned with fluid were considered to be in late proestrus (LP), which is the phase of puberty when LHRH and gonadotropins are discharged for the first time as a preovulatory surge. The first ovulation occurs at first estrus (E) on the following day. At this time, the vagina becomes patent exhibiting predominantly cornified cells, and the ovaries have fresh corpora lutea.

Northern blot analysis

Northern blot hybridization was carried out to determine changes in hypothalamic NELL2 mRNA. Total hypothalamic RNA (20 μg) was separated on a 1.2% formaldehyde-agarose gel, and transferred onto a nylon membrane. The membranes were hybridized with radiolabeled probes in hybridization solution at a specific activity of 1×10^6 cpm/ml. Final posthybridization wash was performed in 0.1 × SSC at 60℃. Autoradiographic images of X-ray film were normalized with densities of 18 S ribosomal RNA bands and calculated as a percent of control value. RNA samples (20 μg) from several tissues including the cerebral cortex, cerebellum, and other peripheral tissues were hybridized with the same cDNA probes, to determine the tissue distribution of the transcripts. NELL2 cDNA probe for northern blot hybridization was labeled with ^{32}P-dCTP (Amersham) using a random primer labeling kit with *Eco*RI digested PCR-cloned cDNA fragment. ^{32}P-labeled probe was

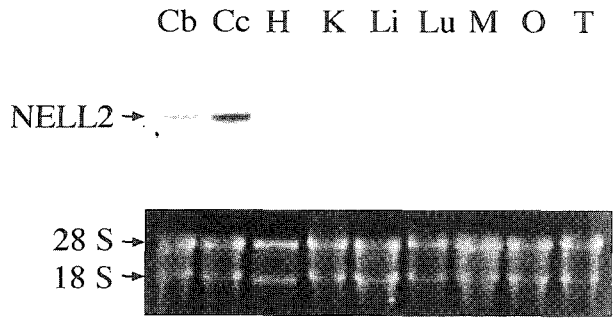

Fig. 1. Brain-specific expression of NELL2. Northern blot analysis shows NELL2 transcript of 3.7 kb in size in the brain areas such as cerebellum (Cb) and cerebral cortex (Cc). No detectable signals were found in the peripheral tissues such as heart (H), kidney (K), liver (Li), lung (Lu), muscle (M), ovary (O) and testis (T). The 18 S and 28 S represent ribosomal RNA bands stained with ethidium bromide.

separated from the free isotope with a Nick column.

Targeted disruption of NELL2 synthesis

To examine the involvement of NELL2 in the regulation of female puberty onset, an antisense oligodeoxynucleotide (ODN) was delivered to the lateral ventricle via a stereotaxically implanted cannula, and the day at which vaginal opening occurred was observed. Antisense NELL2 ODN was designed to have antisense sequence against 21 nucleotides including translation initiation site based on the previous data (Kuroda et al., 1999): antisense NELL2 ODN, 5'-CCG GGA TTC CAT GGC GTG CAT-3'. As control, scrambled (SCR) sequence of antisense NELL2 ODN was used: SCR NELL2 ODN, 5'-TAT CGC ATG CGG GCC TAT GCG-3'. The scrambled sequence did not bear similarities with any eukaryotic sequence thus far deposited in the NCBI GenBank.

Under pentobarbital (7.5 mg/kg BW) and ketamine hydrochloride (25 mg/kg BW) anesthesia, a polyethylene guide cannula (od 1.05 mm, id 0.35 mm) with inner stylet (27 gauge) was stereotaxically implanted into the lateral ventricle (1.1 mm rostral to the bregma, 1.7 mm lateral from the midline, and 4.2 mm vertical from the surface of skull) of 24-day old female SD rats and fixed in place with anchor screws and dental cement. At the end of the recovery period of 4 days, the inner stylet was removed and the ODNs (200 ng/rat, respectively) were i.c.v.-injected with a Hamilton syringe once a day for 2 days. Two days after the second delivery of ODNs, we verified the selective effect of

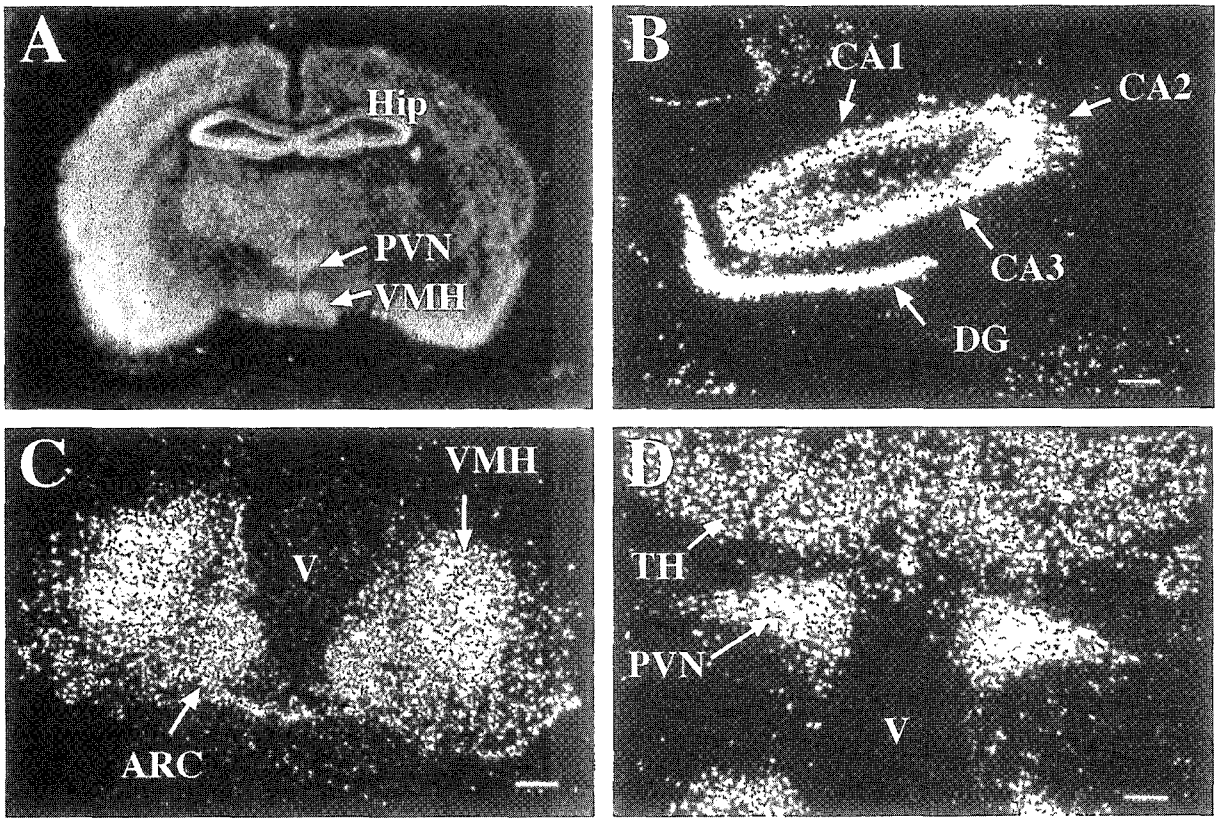

Fig. 2. Localization of NELL2 mRNA in the coronal sections of the female rat brain. Sections were hybridized with ^{35}S-labeled antisense NELL2 RNA probe. Dark field of film autoradiography (A, magnification of 10x) represents the positive signals of NELL2 mRNA in the hypothalamic area including ventromedial hypothalamic nucleus (VMH) and paraventricular nucleus (PVN). Strong signals are also found in the hippocampus (Hip). Dark fields of emulsion autoradiographs show dense signals in CA1, CA2 and CA3 layers of hippocampus, and dentate gyrus (DG) (B). VMH and arcuate nucleus (ARC) in medial hypothalamic area show dense positive signals (C). Thalamus (TH) and PVN also show clear signals of NELL2 mRNA (D). V; third ventricle. Scale bars=100 μm.

antisense ODN on the NELL2 mRNA level in the MBH of some animals using northern blot analysis. The functional consequence of antisense NELL2 ODN on the onset of female puberty was determined by observation of ages at the vaginal opening.

Statistical analysis

The results were analyzed with a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications. Differences between two means were analyzed with the Student t-test.

Results

Tissue-specific expression of NELL2

The present northern blot data showed that NELL2 cDNA probe hybridized with a single transcript of about 3.7 kb (Fig. 1). NELL2 showed tissue-specific expression only among tissues that we selected to determine the transcription in the brain regions (Fig. 1). No signal was observed in the peripheral tissues of 40-day old rats. Brain-specific expression of NELL2 is consistent with previous reports (Matsuhashi et al., 1995; Watanabe et al., 1996; Kuroda et al., 1999; Oyasu et al., 2000), which showed nervous tissue-specific expression of chicken nel, and human and rat NELLs.

Antisense RNA probe complementary to the coding region of NELL2 mRNA was used to determine the cellular sites of NELL2 mRNA expression in the coronal sections of the female rat brain (Fig. 2). Although specific labeling was detected in cells scattered throughout the brain, NELL2 mRNA was more abundant in discrete brain areas including hippocampus and hypothalamic area. NELL2 mRNA was abundant in the hippocampal layers of CA1, CA2 and CA3, and dentate gyrus. No detectable positive signals were observed in the sections hybridized with the sense probe (data not shown).

NELL2 mRNA is very abundant in the hypothalamic areas such as paraventricular nucleus (PVN) and ventromedial nucleus (VMH). The PVN and VMH are located in the medial region of the hypothalamus, lateral to the third ventricle in a dorsal and intermediate position, respectively, along the sides of the third ventricle. Clear positive signals were also found in cells in the arcuate nucleus (ARC), which is located in a ventral position to the third ventricle in the medial region of the hypothalamus. VMH has been known to be responsible for sex behavior and sex differentiation in the mammalian species (Yahr and Ulibarri, 1986; Romano et al., 1990; Nicot et al., 1997; Rachman et al., 1998; Chu and Etgen, 1999). For example, preproenkephalin is highly concentrated in the VMH and its expression is steroid-dependent and involved in the regulation of sex behavior (Romano et al., 1990; Nicot et al., 1997). NELL2 gene expression is regulated by steroid hormone and it is highly expressed in VMH, suggesting that it may be involved in the sex-related function in this brain area.

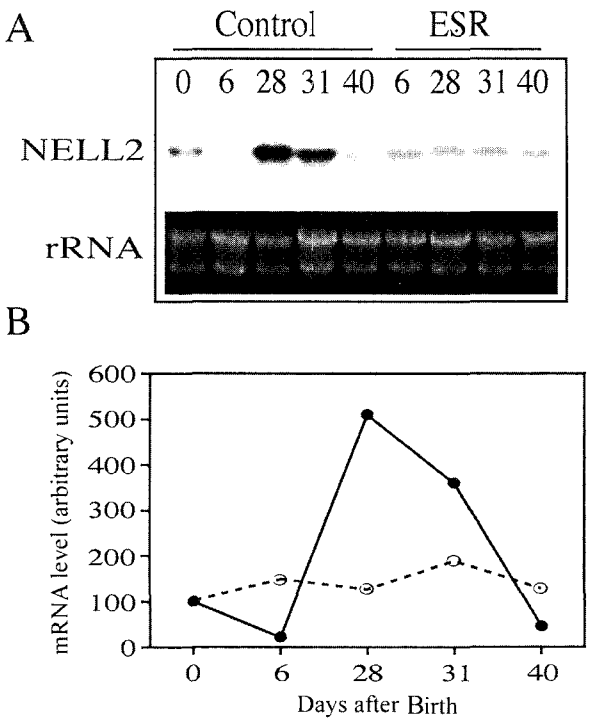

Fig. 3. Postnatal ontogeny of NELL2 mRNA in the rat hypothalamus. RNA samples (20 μg) from estrogen-sterilized rat (ESR) and control rat hypothalamus were northern hybridized with ^{32}P-labeled cDNA probe (A). Densities were scanned, normalized with 18 S ribosomal RNA bands and calculated as percent of control (0-day value). NELL2 mRNA levels show increase at the prepubertal 28-day of age (B). Neonatal treatment of estradiol-benzoate (, ESR) clearly suppressed the prepubertal peaks of mRNA levels observed in the control(●).

Developmental pattern of NELL2 gene expression

We analyzed NELL2 mRNA level during postnatal development with northern blot analysis. Radiolabeled cDNA probe was generated by labeling the *Eco*RI digested insert of pGEM-T easy vector and was hybridized to RNA samples from 0-, 6-, 28-, 31-, and 40-day old ESR and control rat hypothalami. The hybridization bands of the autoradiographs were quantitated by densitometric scanning, followed by normalization with respect to densities of 18 S rRNA bands stained with ethidium bromide and represented as percent of control value.

Northern blot analysis showed that NELL2 mRNA reached peak at 28-day and continued to be high level until 31-day of age, at which it correspond to the late juvenile and early pubertal period, respectively (Fig. 3). Thereafter, the mRNA level decreased at 40-day of age. On the contrary, neonatal treatment of EB ESR significantly suppressed the increases in NELL2 mRNA levels at 28- and 31-day of age. Based on the prepubertal increase in NELL2 mRNA level, we designed to further determine the detailed change in NELL2 mRNA

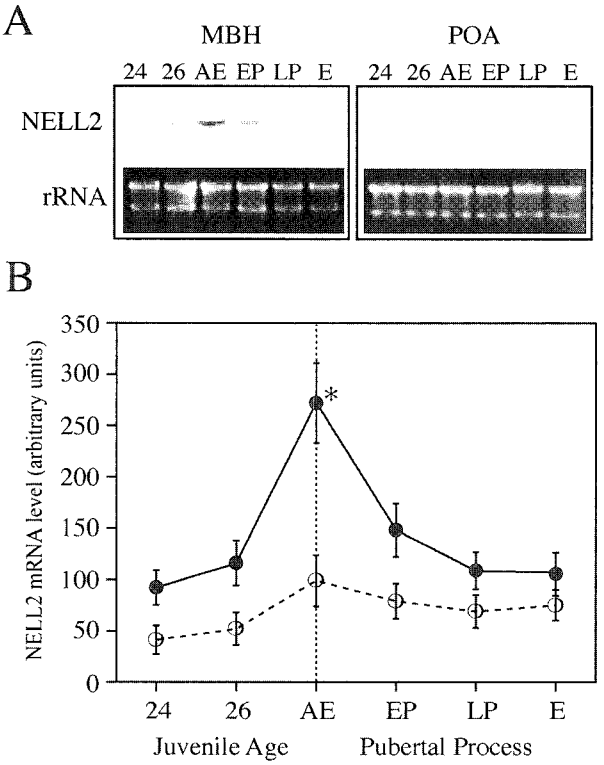

Fig. 4. Change in NELL2 mRNA levels during the pre- and peripubertal period in the female rat hypothalamus. RNA samples from medial basal hypothalamus (MBH) and preoptic area (POA) were hybridized with a ^{32}P-labeled cDNA probe (A). Band densities from 3 repeated experiments were normalized with densities of 18 S ribosomal RNA, calculated and represented as percent of control (24-day MBH value) in format of mean ± SEM. NELL2 mRNA level in MBH(●) peaked at anestrus (AE), while no significant change was observed in POA(○). *=P<0.01 versus other points in MBH tissues. Abbreviations: EP, early proestrus; LP, late proestrus; E, first estrus. Numbers in panel A and X-axis in panel B represent days of age.

levels during the pre- and peripubertal period.

Pubertal change in NELL2 mRNA level

To determine the change in the NELL2 mRNA levels during the pre- and peripubertal period, northern blot analysis was carried out with hypothalamic RNA samples from female rats of 24- and 26-day of age, and showing anestrus (AE), early proestrus (EP), late proestrus (LP), and first estrus (E) (Fig. 4). NELL2 mRNA in the MBH tissue showed a dramatic peak at AE, and thereafter it decreased to the level observed at the late juvenile age (26-day of age). Its level in POA did not show such a dramatic change during the same period. The prepubertal change in the NELL2 mRNA level in MBH suggests a possible role of NELL2 in the process leading to the onset of female puberty.

Effect of antisense NELL2 oligodeoxyncleotide (ODN) on the puberty initiation of female rats

To block synthesis of NELL2, we introduced antisense NELL2 ODN into the lateral ventricle of the female rats

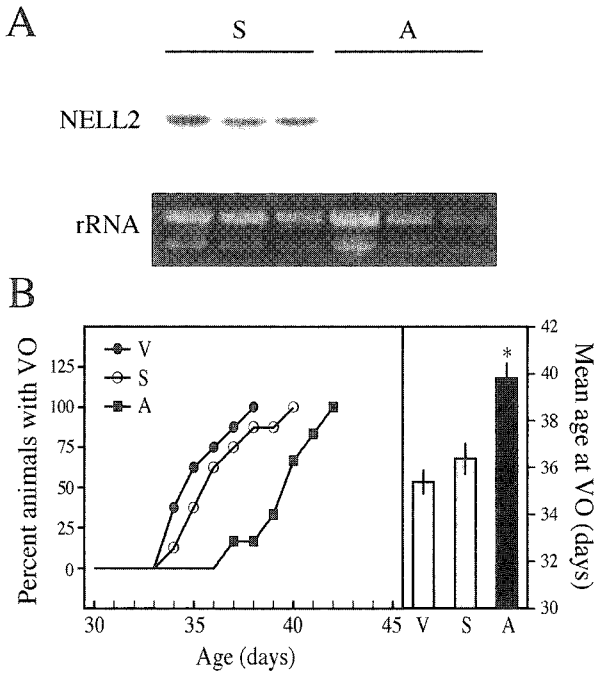

Fig. 5. Effect of targeted disruption of NELL2 synthesis on the onset of female puberty. The antisense (A) or its scrambled (S) ODN were infused into the lateral ventricle of the brain once a day for 2 days (28- and 29-days of age), and RNA samples from some rats were examined with northern blot analysis to validate the effect of antisense ODN (A). Antisense ODN induced degradation of NELL2 mRNA, which resulted in smear hybridization bands. Panel B shows percent animals showing vaginal opening (VO) on the day of observation and mean age at VO for each experiment groups. *=P<0.01 versus 2 controls of vehicle (V) and S.

at 28- and 29-day of age. Two days later at 31-day of age, we determined mRNA levels of NELL2 to validate the selective effect of ODN on the NELL2 synthesis. Interestingly, NELL2 mRNA signals from antisense NELL2 ODN-injected rat MBH showed smeared bands, suggesting that the antisense NELL2 ODN may induce degradation of NELL2 mRNA (Fig. 5A). Administration of ODN resulted in marked delay of puberty onset, as determined by the ages at vaginal opening compared to the sham-operated and SCR-injected controls (Fig. 5B). The functional consequence of the antisense ODN showing the delay of vaginal opening further suggests that NELL2 plays an important role in the regulation of the onset of female puberty.

Discussion

The identification of genes involved in the regulation of puberty is important for understanding of the development and differentiation of sex-related brain function. In this study, we focused on determination of a possible involvement of a gene, NELL2, in the female puberty. NELL2 has been known as a neural cell-specific protein suggested to play an important role in the development and differentiation of nerve cells (Kuroda et al., 1999; Kuroda and Tanizawa, 1999; Oyasu et al., 2000). We found, using ddPCR and low density cDNA

array, that NELL2 expression is up-regulated in the adult female rats that neonatally received high dose of EB (Choi and Lee, 1999; Ha and Lee, 2000). NELL2 mRNA level was also regulated by neonatal ovariectomy (OVX) and EB or T replacement (Ha and Lee, 2000). Therefore, NELL2 may be a new steroid-responsive gene, although information about its 5' flanking region of is not so far available. The neonatal treatment of a high dose of EB has been known to block the normal differentiation of sex-related hypothalamic anatomy and function (Barraclough, 1961; Gorski, 1963; Hayashi and Aihara, 1989; Hayashi et al., 1991). Moreover, the NELL2 mRNA level showed a significant change in a steroid treatment regime such as neonatal OVX and EB or T replacement that can modulate brain sex differentiation (Doughty et al., 1975; Jacobson et al., 1981; Register et al., 1995). Therefore, we hypothesized that NELL2 might be involved in the hypothalamic sex differentiation, which is necessary for the normal puberty initiation in female.

NELL2 was confirmed to play an important role in female puberty initiation, because the administration of antisense NELL2 ODN clearly delayed vaginal opening, a criteria for puberty initiation. It has been assumed that the action of antisense ODN is mediated through a blockade of mRNA translation and, possibly through the degradation of the target mRNA by RNase H at the level of ODN-mRNA hybrids (Wahlestedt, 1994; Landgraf, 1996; Nicot and Pfaff, 1997). The present results showed mRNA degradation by the antisense ODN treatment, indicating that its site of action is before translation initiation. It is presumed that the antisense ODN acts on NELL2 synthesis by inducing mRNA digestion with RNase H at the site of ODN-mRNA hybrid. However, the antisense ODN could also block translation itself (Nicot and Pfaff, 1997), and the actual level of NELL2 protein in the antisense ODN-treated rat hypothalamus could be much lower than expected from the present northern blot data.

The detailed mechanism of NELL2 function in the regulation of female puberty is unclear thus far. Recently, NELL2 has been identified as a protein kinase C (PKC) binding protein and to be phosphorylated by PKC (Kuroda et al., 1999). EGF-like domains of NELL2 were known to be involved in the isoform-specific interaction with PKC. Therefore, it is presumed that PKC-dependent phosphorylation of NELL2 may be responsible for the process leading to the neuronal events of female puberty in the neuronal cells in hypothalamic nuclei such as VMH. NELL2 has other functional domains of thrombospondin-1 (TSP-1)-like module in its N-terminal region (Beckmann et al., 1998) and five von Willebrand factor (vWF) C domains (Hunt and Barker, 1987; Bork, 1993). With the presence of TSP-1 module, NELL2 was suggested to be at least partly secreted from the COS-7 cells expressing NELL2 and interact with heparan sulfate proteoglycans (Kuroda et al., 1999). The vWF domains are presumed to play a role in the homotrimeric association of NELL2. NELL2 may play a role as a signaling molecule and may be partly responsible for the neuronal event before puberty onset.

The NELL2 immunoreactivity and mRNA was widely distributed throughout the brain according to our in situ hybridization analysis and the previous report (Oyasu et al., 2000). The highest densities of NELL2 immunoreactivity and mRNA were seen in the hippocampus, while the hypothalamus showed relatively low density of mRNA and immunoreactivity. Among the hypothalamic nuclei, VMH, which is important for regulating sexual behavior, showed the strongest NELL2 mRNA signals. In the neuronal cells, NELL2 proteins were reported to be located in the rough endoplasmic reticulum and suggested to play a role in regulating protein synthesis (Oyasu et al., 2000). Therefore, this could also be a possible mechanism of NELL2 action for regulating neuronal process for puberty onset.

Although the precise biological role of NELL2 has not yet been assigned, our recent data showed that antisense NELL2 ODN markedly suppressed mRNA levels of sex-related genes such as progesterone receptor and preproenkephalin (Ha and Lee, 2000), suggesting that NELL2 may play an important regulatory role on the sexual differentiation of hypothalamus. No data are available so far about the 5' flanking sequence of NELL2. Therefore, we are not sure about whether E directly regulates the NELL2 gene expression through binding to the E-responsive element of 5' flanking region or indirectly through other E-responsive gene products. However, NELL2 gene expression was dependent upon steroid environment according to our previous data (Choi and Lee, 1999) and northern blot analysis in the present study. Therefore, NELL2 may be responsible for the puberty initiation mechanism that occurs in the hypothalamus under the steroid feedback environment.

Taken together the present study showed that a new E-responsive gene NELL2 is important in the puberty initiation.

Acknowledgements

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