Journal of Chemical Neuroanatomy 43 (2012) 28–33

Contents lists available at SciVerse ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Immunolocalization of steroidogenic acute regulatory protein-related lipid transfer (START) domain-containing proteins in the developing cerebellum of normal and hypothyroid rats

In Youb Chang [a,1], Takbum Ohn [b,1], Gil Seok Ko [a], Young Yoon [c], Jung Woo Kim [d], Sang Pil Yoon [e,*]

[a] Department of Anatomy, College of Medicine, Chosun University, Gwangju, Republic of Korea
[b] Division of Natural Medical Sciences, Chosun University, Gwangju, Republic of Korea
[c] Department of Optometry, Daebul University, Jeonnam, Republic of Korea
[d] Department of Anatomy, College of Medicine, Seonam University, Namwon, Jeollabuk-Do, Republic of Korea
[e] Department of Anatomy, School of Medicine, Jeju National University, 66 Jejudaehakno, Jeju-Si, Jeju-Do 690-756, Republic of Korea

ARTICLE INFO

Article history:
Received 15 June 2011
Received in revised form 10 October 2011
Accepted 10 October 2011
Available online 17 October 2011

Keywords:
Development
Propylthiouracil
Purkinje cells
StAR
StarD6

ABSTRACT

Cholesterol transport proteins are a prerequisite for neurosteroidogenesis. Steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain-containing proteins, such as StAR and START domain-containing 6 (StarD6), are known to be distributed in the brain. Since perinatal hypothyroidism affects cerebellar development, we examined postnatal changes in StAR and StarD6 immunolocalization in the developing cerebellum of control and hypothyroid rats. Pregnant Sprague-Dawley rats were given 0.05% 6-propyl-2-thiouracil (PTU) or water from gestation day 11 until postnatal day (P) 28, and were then killed together with age-matched control rats. As shown by calbindin D-28k immunostaining, the developing cerebellar cytoarchitecture and Purkinje cells were affected by PTU-induced hypothyroidism as compared to control rats. The immunolocalization of StAR and StarD6 generally followed the maturation pattern of Purkinje cells from the vermis to the cerebellar hemisphere. StAR immunostaining first appeared in the Purkinje cells of the vermis at P7 in both control and hypothyroid rats. In control rats, a few StarD6 immunoreactive cells were seen at birth and a nuclear localization of StarD6 in Purkinje cells was obvious at P14. PTU-induced hypothyroidism delayed the appearance of StarD6 immunopositive cells until P7. Moreover, the nuclear localization of StarD6 in PTU-treated rats was not obvious at P14. An adult-like distribution of StAR and StarD6 was achieved by P21 in control and hypothyroid rats. These results suggest that StarD6 may affect the development of Purkinje cells during the first and second postnatal weeks, a known period of thyroid hormone action.

1. Introduction

Thyroid hormone (TH) is essential for normal brain development and plays important roles in cell proliferation, migration, synaptogenesis, and myelination in the developing brain (Anderson et al., 2003). The fetal brain is sensitive to maternal thyroid status (Morreale de Escobar et al., 2004; Pemberton et al., 2005), and maternal hypothyroxinemia can potentially cause neuronal damage in the fetus. The adverse consequences of this depend on its severity and the developmental stage at which it occurs. Doses of 6-propyl-2-thiouracil (PTU) that cause TH insufficiency adversely affect the developing brain (Zoeller and Crofton, 2005).

The developing cerebellum is vulnerable to TH deficiency (Anderson, 2008; Koibuchi, 2008). PTU-treated rats show growth retardation, a reduction in cerebellar mass, and alterations in cerebellar structure (Li et al., 2004), which are characterized by delayed migration of granular cells, short Purkinje cell dendritic arborization, and a significant reduction in the number of synaptic connections. PTU also modulates the integrity of neural cytoskeleton components such as neurofilament (Zamoner et al., 2008; Chinnakkaruppan et al., 2009), microtubule (Benjamin et al., 1988), and calbindin D-28k (CB, Saegusa et al., 2010).

De novo synthesis of neurosteroids from cholesterol is known to occur in the cerebellum, mainly in the Purkinje cells (Tsutsui, 2006), which actively synthesize progesterone during neonatal life, when cerebellar cortical formation occurs. Neurosteroids including progesterone, estrogen, and allopregnenolone may promote dendritic growth, spinogenesis, and synaptogenesis in Purkinje cells (Sakamoto et al., 2001, 2002). Neurosteroids are considered to be neuroprotective (Wojtal et al., 2006), and inhibition of

* Corresponding author. Tel.: +82 64 7543823; fax: +82 64 7252593.
E-mail address: spyoon@jejunu.ac.kr (S.P. Yoon).
[1] These authors contributed equally to this work.

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doi:10.1016/j.jchemneu.2011.10.003

neurosteroidogenesis increases apoptosis in the molecular and granular layers of the cerebellum, and also increases the number of pyknotic Purkinje cells (Yawno et al., 2009).

The first step in the biosynthesis of neurosteroids is the conversion of cholesterol to pregnenolone, and the transport of cholesterol to mitochondria is a prerequisite for neurosteroidogenesis (Wojtal et al., 2006). The cholesterol transport protein steroidogenic acute regulatory protein (StAR) is the prototypic StAR-related lipid transfer (START) domain-containing proteins (Alpy and Tomasetto, 2005). A general trend toward lower levels of neurosteroids is observed in patients with Alzheimer's disease (Schumacher et al., 2003), while levels of StAR are elevated in these patients (Webber et al., 2006) as well as in aged rats (Sierra et al., 2003).

START domain-containing 6 (StarD6) is first reported in the male gonad (Gomes et al., 2005; Chang et al., 2007a). We reported that StarD6 is distributed in the rat nervous system (Chang et al., 2007b) and responds to excitotoxic brain injury (Chang et al., 2009). Morphological aspects of StarD6 differ from those of StAR (Chang et al., 2010), although StarD6 has a protease-sensitive C-terminus similar to that of StAR (Bose et al., 2008). While a few reports have been published on StAR (Kim et al., 2002; Sierra et al., 2003; Lavaque et al., 2006), developmental changes in StarD6 have not been reported. The neonatal rat cerebellum would be a useful model, since perinatal hypothyroidism dramatically affects cerebellar development. We examined the postnatal changes in StAR and StarD6 immunolocalization during cerebellar development in normal and PTU-induced hypothyroid rats.

2. Materials and methods

2.1. Animals and treatments

Pregnant Sprague-Dawley rats were purchased from Da-mool Science (Daejeon, Republic of Korea) on gestation day (GD) 9; the day of breeding being taken to be GD 0. Six pregnant rats per group were given water (control) or 0.05% (w/v) PTU (Antiroid™, Bukwang Pharm. Co., Seoul, Republic of Korea) orally on a daily basis from GD 11 to postnatal day (P) 28, as described in the procedure previously described by Chang et al. (2007a), with slight modifications. Animals were divided into control and PTU-induced hypothyroid groups, and changes in body weight were checked every postnatal week throughout experimental period. Animals were killed on P0, P7, P14, P21, and P28 ($n = 5$/each age), respectively. All experimental procedures and care of animals were conducted in accordance with the guidelines of Jeju National University's Animal Care and Use Committee.

2.2. Immunohistochemistry

After fixation with 4% paraformaldehyde, the cerebellum was embedded in paraffin wax (Tissue-Tek, Sakura, Japan) by a standard procedure. Next, 5-μm-thick serial sections were cut using a RM 2155 rotary microtome (Leica Microsystems, Nussloch, Germany) and mounted on slides coated with 3-aminopropyl-tri-ethoxy-silane (Sigma–Aldrich, St. Louis, MO).

Immunohistochemical staining was carried out using a routine method. Briefly, tissue sections were incubated at 4 °C for 24 h with primary antibody: monoclonal anti-StAR (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or the polyclonal anti-StarD6 (kindly supplied by Dr. Jaemog Soh, Chonnam National University, Gwangju, Republic of Korea). Antibody binding was visualized using an ImmPRESS™ avidin–biotin-peroxidase kit (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's instructions. Omission of incubation with the primary or secondary antibody was used to control for false-positives. Images were captured directly using an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan) and a C-4040Z digital camera (Olympus Corp.). The captured images were saved and subsequently processed using Adobe Photoshop (Adobe System, San Jose, CA). The brightness and contrast of the images were adjusted only for the purpose of background consistency.

2.3. Statistical analysis

Body weights are expressed as the mean ± SD. Statistically significant differences in weight gain between control and hypothyroid rats was assessed by the generalized linear model method ANOVA with repeated measures, and differences between groups at the same age were evaluated by Student's *t*-test. All statistical analyses were performed using SPSS version 12.0 (SPSS, Chicago, IL). A p value of <0.05 was deemed to be statistically significant.

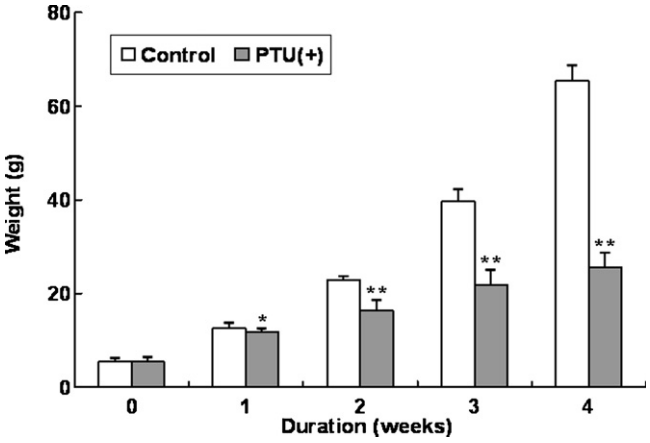

Fig. 1. Postnatal weight gain in control and 6-propyl-2-thiouracil (PTU)-induced hypothyroid rats. PTU treatment (PTU+) caused growth retardation and resulted in a growth curve that was considerably different compared to that of control rats ($p < 0.01$). A significant difference was noted between control and PTU-treated hypothyroid rats of the same age ($^{*}p < 0.05$, $^{**}p < 0.01$).

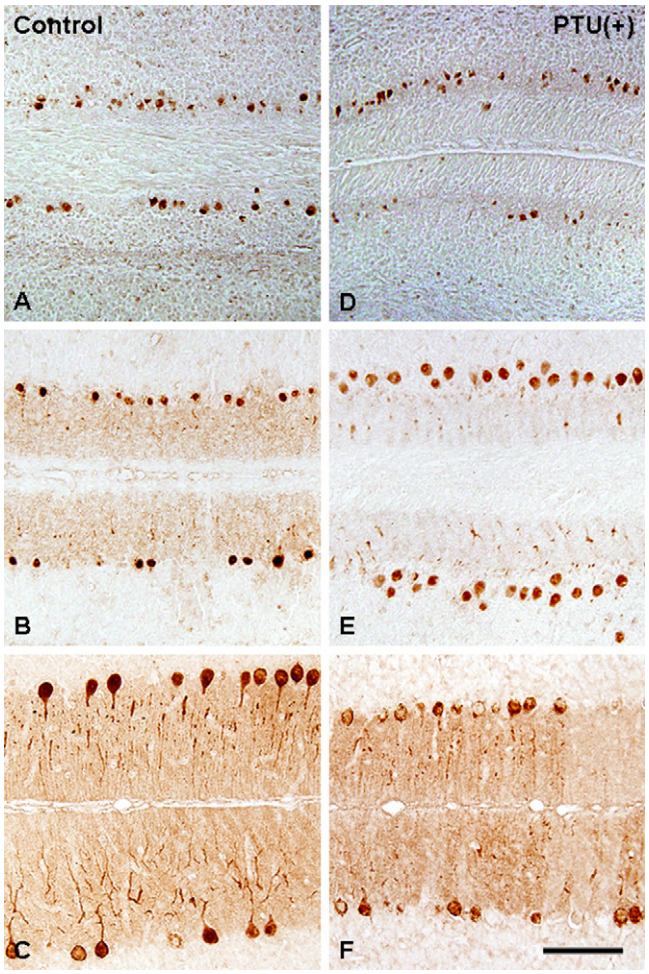

Fig. 2. Calbindin D-28k (CB) immunostaining in the developing cerebellum. Cerebellar CB staining was evaluated on postnatal days (P) 7 (A and D), 14 (B and E), and 28 (C and F), ages at which considerable differences were observed between the control and 6-propyl-2-thiouracil (PTU)-treated rats. Cerebellar CB immunoreactivity was delayed and reduced at all ages in PTU-treated rats compared to control rats. Note the multiple layers of Purkinje cells and short dendritic arborization at P14 (E) in hypothyroid rats. Scale bar = 100 μm.