Tissue and Cell 49 (2017) 514–519

Contents lists available at ScienceDirect

Tissue and Cell

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

NeuN immunoreactivity in the brain of *Xenopus laevis*

Shouichiro Saito[a,*], Takahiko Shiina[b], Yasutake Shimizu[b]

[a] Laboratory of Veterinary Anatomy, The United Graduate School of Veterinary Science, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan
[b] Laboratory of Veterinary Physiology, The United Graduate School of Veterinary Science, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan

ARTICLE INFO

Article history:
Received 6 April 2017
Received in revised form 28 April 2017
Accepted 3 May 2017
Available online 13 May 2017

Keywords:
Antigen retrieval
Fox-3
Phylogeny

ABSTRACT

Neuronal nuclear antigen (NeuN), discovered in mice brain cell nuclei by Mullen et al. (1992), is used as an excellent marker of post-mitotic neurons in vertebrates. In this study, the expression pattern of NeuN was examined in the *Xenopus* brain to explore phylogenetic differences in NeuN expression. Anti-NeuN antibody showed selective staining in mouse and *Xenopus* brain extracts, but the number and molecular weight of the bands differed in Western blotting analysis. In immunostaining, anti-NeuN antibody showed selective staining of neurons, but not glial cells, in the *Xenopus* brain. Most neurons, including olfactory bulb mitral cells and cerebellar Purkinjie cells, which show no immunoreactivity in birds/mammals, showed NeuN immunoreactivity in *Xenopus*. This study revealed that anti-NeuN antibody is a useful marker of post-mitotic neurons in amphibians, but it also stains neurons that show no reactivity in more derived animals.

1. Introduction

Neuronal nuclear antigen (NeuN) is a protein discovered using a monoclonal antibody in mice brain cell nuclei (Mullen et al., 1992), and various kinds of post-mitotic neurons in the central and the peripheral nervous system have been shown to express NeuN (Wolf et al., 1996; Sarnat et al., 1998; Weyer and Schilling 2003; Nassauw et al., 2005; Lavezzi et al., 2013). In Western blotting, anti-NeuN antibody revealed bands at approximately 46 and 48 kDa in the mammalian brain (Mullen et al., 1992), and these two isoforms are thought to be different phosphorylated forms (Lind et al., 2005) and/or alternative splicing forms (Kim et al., 2009). Both isoforms, which are localized in both neuronal nuclei and the cytoplasm (Lind et al., 2005), were identified as a Fox-3 gene product, a neuron-specific splicing regulator (Kim et al., 2009). Also in Western blotting of mammalian brain extract, anti-NeuN antibody has an additional weak band of approximately 70 kDa (McPhail et al., 2004; Ünal-Cevik et al., 2004; Lind et al., 2005). This band corresponds to synapsin I, a neuron-specific synaptic vesicle-associated phosphoproteins. In immunohistochemistry, anti-NeuN antibody appears to be unable to detect synapsin I, because of its low affinity against it (Kim et al., 2009).

NeuN is widely accepted as a representative maker of post-mitotic neurons. On the other hand, Mullen et al. (1992) also found in the adult mouse that anti-NeuN antibody showed no immunoreactivity in mitral cells of the olfactory bulb, cerebellar Purkinjie cells, retinal photoreceptor cells, and a population of large neurons in the dorsal cochlea nucleus. Sarnat et al. (1998) demonstrated

Abbriviations: A, anterior thalamic nucleus; Ad, anterodolsal tegmental nucleus; AL, lateral amygdala; AM, medial amygdala; Av, anteroventral tegmental nucleus; C, central thalamic nucleus; Cer, cerebellar nucleus; Cll, caudal nucleus of lateral line nerve; DH, dorsal hypothalamic nucleus; DP, dorsal pallium; DS, dorsal striatum; Ea, anterior entopeduncular nucleus; Ep, posterior entopeduncular nucleus; Gc, gray layer of rhombencephalon; GL, olfactory bulb granular cell layer; Gran, cerebellum granular cell layer; GT, trigeminal ganglion; Hd, dorsal habenular nucleus; Hv, ventral habenular nucleus; Is, isthmic nucleus; L, lateral thalamic nucleus; LP, lateral pallium; LS, lateral septum; ML, olfactory bulb mitral cell layer; MP, medial pallium; MS, medial septum; NA, accumbens nucleus; NC, cuneate nucleus; Ng, gracile nucleus; nV, trigeminal nerve; Ols, superior olivary nucleus; P, posterior thalamic nucleus; Pd, posterodorsal tegmental nuclues; PE, postolfactory eminence; Po, preoptic nucleus; PT, posterior tuberculum; Purk, Purkinjie cell layer; Pv, posteroventral tegmantal nucleus; Rad, raphe nucleus, dorsal part; Ri, inferior reticular nucleus; Ris, isthmic reticular nucleus; Rll, rostral nucleus of lateral line nerve; Rm, medial reticular nucleus; SC, suprachiasmatic nucleus; Sol, nucleus of solitary tract; Tel, telencepharon; TM, gray layer of tectum of mesencephalon; TO, olfactory tubercle; Tp, principal nucleus of semicircular torus; VH, ventral hypothalamic nucleus; VLd, ventrolateral thalamic nucleus, dorsal part; VLv, ventrolateral thalamic nucleus, ventral part; VM, ventromedial thalamic nucleus; VS, ventral striatum; III, nucleus of oculomotor nerve; IV, nucleus of trochlear nerve; Vd, descending nucleus of trigeminal nerve; Vm, motor nucleus of trigeminal nerve; Vpr, principal nucleus of trigeminal nerve; VIIm, motor nucleus of facial nerve; VIIId, dorsal nucleus of cochlea nerve; VIIIv, ventral nucleus of cochlea nerve; IX, nucleus of glossopharyngeal nerve; Xm, motor nucleus of vagus nerve.

* Corresponding author.
E-mail address: shouichi@gifu-u.ac.jp (S. Saito).

http://dx.doi.org/10.1016/j.tice.2017.05.001
0040-8166/

that no NeuN-immunoreactivity was observed in the inferior olivary nuclei of the brainstem, Purkinjie cells and dentate nucleus of the cerebellum, and Cajal-Retzius neurons of the cerebral cortical plate, in the human fetal brain. Weyer and Schilling (2003) demonstrated that basket and stellate cells, Golgi neurons, unipolar brush cells, and Lugaro cells in developing and adult mouse cerebella showed no immunoreactivity for anti-NeuN antibody. Interestingly, Kumar and Buckmaster (2007) revealed the absence of NeuN-immunoreactivity in the substantia nigra pars reticulata in the gerbil, whereas intense immunoreactivity was observed in the rat. This finding suggests species-specific differences in NeuN immunoreactivity in certain types of neurons among vertebrate species. With regard to NeuN immunoreactivity and phylogeny, Mullen et al. (1992) reproted cross reactivity of anti-NeuN antibody to neurons in chickens, humans, and salamanders. Furthermore, NeuN immunoreactivity was found in larvae and froglets of the amphibian *Xenopus laevis* by Yoshino and Tochinai (2004, 2006), and interestingly, NeuN immunoreactivity could be seen in olfactory bulb mitral cells in this species (Yoshino and Tochinai, 2006), whereas no NeuN immunoreactivity was found in mammals. To clarify phylogenetic differences in NeuN expression, this study examined NeuN immunoreactivity in the brain of adult *Xenopus laevis*.

2. Materials and methods

2.1. Animals

Ten male adult *Xenopus laevis* were purchased from Hamamatsu Biological Materials (Shizuoka, Japan). Seven animals were used for immunohistochemistry, and three for Western blotting. One 10-week-old male ddY mouse obtained from Japan SLC (Shizuoka, Japan) was also prepared for Western blotting. For immunohistochemistry, after anesthesia by intraperitoneal injection of 5.0×10^{-3} ml/g body weight of sodium pentobarbital (Abbott, North Chicago, IL, USA), animals were euthanized by cardiac perfusion with physiological saline and 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The brains were removed, immersed in the same fixative for 24 h, and embedded in paraffin. Serial paraffin sections were cut coronally at 5-μm in thickness, mounted on slides coated with poly-L-lysine, and processed for immunohistochemistry. For Western blotting, after anesthesia as described above, the brain, kidney and small intestine were quickly removed and processed. All procedures were in accordance with the Guide for Care and Use of Animal Experimentation at Gifu University, Japan (Permission No. 13008).

2.2. Western blotting

The brain, kidney, and small intestine from *Xenopus* and the mouse brain were homogenized in CelLytic reagent (Sigma-Aldrich Co., St. Louis, MO, USA), and the supernatant was obtained from each homogenate by centrifugation at 12,000*g* for 10 min. Protein concentration of each supernatant was determined by the Bradfold assay using the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein samples were mixed 2:1 with 3 × SDS-PAGE sample buffer (24% glycerol, 4.8% SDS, 0.06% bromphenol blue, 3% ß −mercaptoethanol in 144 mM Tris-HCl buffer, pH 6.8) and heated at 95 °C for 5 min. For each sample, a total of 10 μg of protein were loaded onto each well of an 8% SDS-PAGE gel and electrophoresed. The proteins were then transferred to a PVDF membrane by semidry electroblotting at 100 mA for 60 min. The membrane was incubated in PBST (phosphate-buffered saline containing 0.02% Tween 20) supplemented with 5% nonfat dry milk for 60 min, followed by incubation in anti-NeuN antibody (MAB377, Chemicon International Inc., CA, USA) diluted 1:500 with PBST containing 5% nonfat dry milk for 60 min. After rinsing in PBST three times for 10 min each, the membrane was incubated in peroxidase-conjugated goat anti-mouse IgG diluted 1:2500 with PBST containing 5% nonfat dry milk for 60 min. After another rinsing in PBST, the membrane was colorized in PBST containing 0.02% 3-3′ diaminobenzidine tetrahydrochloride (DAB) and 0.003% H_2O_2.

2.3. Immunostaining

After deparaffinization, the sections were immersed in 0.3% H_2O_2 in methanol at RT for 30 min to eliminate endogenous peroxidase, followed by rinsing in phosphate-buffered saline (PBS, pH 7.4). The sections were then immersed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave at 500W once for 5 min and twice for 1 min 30 s each, with 10 min intervals between heating. This heating condition allowed the buffer solution to be just short of boiling in our microwave treatment. After the buffer solution cooled, the sections were incubated in 0.05 M Tris-buffered saline at RT for 10 min, and rinsed in PBS three times for 5 min each. The sections were then incubated with 2% normal goat serum at RT for 30 min. After rinsing in PBS, the sections were incubated with anti-NeuN monoclonal antibody diluted 1:100 or with anti-GFAP polyclonal antibody (Invitrogen, CA, USA) diluted 1:100 at 4 °C overnight. After rinsing in PBS, the sections were incubated with biotinylated goat anti-mouse IgG for NeuN or biotinylated goat anti-rabbit IgG (Chemicon) for GFAP diluted 1:500 at RT for 30 min. After rinsing in PBS, the sections were incubated with Vectastain ABC reagent (Vector, Burlingame, CA, USA) at RT for 30 min. Finally, the sections were colorized for 10 min in 0.1 M Tris-HCl, pH 7.4, containing 0.02% DAB and 0.003% H_2O_2, then dehydrated and coverslipped. Negative controls were created using PBS to replace either the primary or secondary antibodies. No specific staining was observed in the control sections.

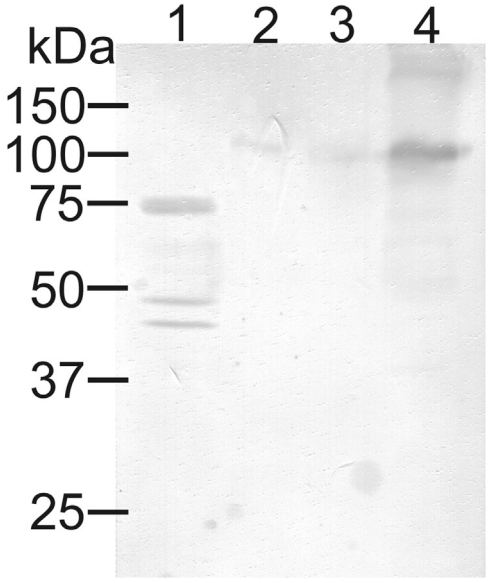

Fig. 1. Western blotting of total protein extracts from mouse brain (lane 1), *Xenopus* kidney (lane 2), *Xenopus* intestine (lane 3), and *Xenopus* brain (lane 4) using anti-NeuN antibody. In the mouse brain, three bands with approximate masses of 75, 46, and 40 kDa were observed. In *Xenopus*, no bands were found in the kidney and intestine, but a single immunoreactive band with an approximate mass of 100 kDa was observed in the brain.