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Neuron-Specific Nuclear Antigen NeuN Is Not Detectable In Gerbil Substantia Nigra *Pars Reticulata*

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Abstract

NeuN (Neuronal Nuclei), the neuron-specific marker of nuclear protein is used extensively in histological procedures to identify major cell-types in adult vertebrate nervous systems of a variety of species including rodents and humans. Some notable exceptions (i.e., NeuN-negative neurons) include Purkinje cells in cerebellum, mitral cells in olfactory bulb, and photoreceptors in retina. Here we report that neurons in gerbil (*Meriones unguiculatus*) substantia nigra *pars reticulata* (SNr), whose “neuronal” phenotype was confirmed via electrophysiology, biocytin-labeling, histology, and *in situ* hybridization, are also devoid of NeuN-immunoreactivity as assayed with the widely used monoclonal antibody A60. Immunohistochemistry of rat SNr using the same antibody yielded robust staining. These data suggest lack of NeuN-immunoreactivity observed in certain cell-types and brain regions can be species-specific.

Keywords

NeuN-immunoreactivity; Substantia Nigra pars reticulata; GABAergic neurons

1. Introduction

NeuN is a neuron-specific nuclear protein expressed in the nucleus and cytoplasm of most neuronal cell types in vertebrate nervous systems. Because of the specificity of its expression, it has come to be regarded as a universal marker for the post-mitotic neuronal phenotype and is used to distinguish neurons from glial and other cells that do not express NeuN. The monoclonal antibody (mAb) A60 generated against brain cell nuclei [17] recognizes NeuN in most neuronal cell-types throughout the nervous system with limited exceptions. These include cerebellar Purkinje cells, mitral cells in olfactory bulbs, retinal photoreceptors, sympathetic chain ganglion cells, Cajal-Retzius cells of neocortex, inferior olivary neurons, and immunocytochemically-identified cerebellar interneurons, which comprise basket and stellate cells, Golgi neurons, unipolar brush cells, and Lugaro cells [17,22,26,27]. The murine epitope of NeuN recognized by mAb A60 has been reported to be conserved among a variety of vertebrate species including birds, rodents, and humans [22]. To our knowledge there are no reports of species-specific differences in NeuN-immunoreactivity. Barring these exceptions,

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NeuN has become a widely accepted immunohistochemical marker for neurons in a variety of tissue culture systems, human and non-human cell lines, and brain sections, so much so that we attempted to use it to identify substantia nigra *pars reticulata* (SNr) neurons in studies of the Mongolian gerbil (*Meriones unguiculatus*), a model of inherited epilepsy [14].

SNr is a predominantly GABAergic nucleus [20] closely apposed to the dopamine-rich *pars compacta* (SNc). SNr is one of two primary output nuclei of the basal ganglia and is involved in orientation and control of eye movements [9]. SNr neurons are believed to play a critical role in modulation of seizures [10,25]. GABAergic neurons of SNr project to and inhibit portions of thalamus and brainstem (superior colliculus and pedunclopontine nucleus) as well as provide local recurrent inhibition to neurons within SNr itself. Dopaminergic neurons of SNc also receive GABAergic input from SNr [7]. However, we found that unlike neurons of SNc, SNr neurons lack NeuN-immunoreactivity in gerbils. To test whether lack of NeuN-immunoreactivity was specific to gerbils and determine whether the phenotype was related to seizures, we evaluated epileptic and non-epileptic gerbil strains and repeated the staining protocol using brain tissue from rats of comparable age. SNr neurons in rat were robustly labeled, unlike those in either epileptic or non-epileptic gerbils, suggesting a species-specific difference in NeuN expression.

2. Results

SNr neurons are NeuN-positive in rat but not gerbil

Immunohistochemistry with NeuN mAb A60 in rat showed robust labeling of SNr neurons in all sections obtained from 4 animals (Fig. 1A1–A2). Fig. 1A2 is a magnified view of the boxed region in Fig. 1A1 showing overall patterns of NeuN-labeling within SNr and adjoining SNc. Nissl-staining (0.25% thionin) of corresponding sections taken from the same animal clearly demarcates a sparsely nucleated SNr from SNc, which is a more densely-packed strip of neurons bordering SNr (Fig. 1A3). SNr is littered with numerous cell bodies sparsely distributed throughout the nucleus without any apparent laminar-specificity.

In sharp contrast with rat, immunohistochemistry with NeuN mAb A60 in gerbils showed little or no immunoreactivity in SNr (n = 6 animals of both genders belonging to both epileptic and non-epileptic strains, Fig. 1B1–B2). In comparison with SNr, SNc and other brain stem nuclei were intensely NeuN-positive (Fig. 1B1). Higher magnification revealed that although a vast majority of cells in SNr were devoid of NeuN-immunoreactivity, on rare occasions we observed within SNr several neurons with large cell-bodies that had intense NeuN-labeling, similar to neurons within SNc (Fig. 1B2). These may be dopaminergic [6,11,14,21], although the precise cellular identity remains to be determined. To confirm that lack of staining in the gerbil SNr did not simply reflect lack of neurons, we examined adjacent Nissl-stained sections from the same animals (Fig. 2B3). Staining patterns were consistent in all series of sections examined, with numerous cell bodies in both SNr and SNc, comparable to those seen in rat (Fig. 1A3 versus Fig. 1B3).

Other neuronal markers are normal in gerbil SNr

To determine cellular composition and ascertain whether commonly known anatomical features were expressed in SNr of gerbils, adjacent sections were processed for tyrosine hydroxylase-immunoreactivity or GAD65 mRNA *in situ* hybridization (Fig. 2). SNr could be easily identified on Nissl-stained sections as a sparsely nucleated region bordering a more densely packed strip of neurons, the SNc (Fig. 2A). Higher magnification revealed numerous well-defined intensely Nissl-stained cell bodies within SNr. This suggests there is no paucity of neurons in the gerbil SNr. To further demarcate GABA-rich SNr from dopamine-rich SNc, we used adjacent sections from the same animals to stain for tyrosine hydroxylase (TH), the

rate-limiting enzyme in dopamine synthesis (Fig. 2B). As expected, TH labeling was concentrated in SNc, with intense staining of both cell bodies as well as dendrites that projected deep into SNr [2]. Few, if any, cell bodies in SNr were TH-positive. Conversely, *in situ* hybridization for GAD65, an enzyme that makes GABA, labeled cell bodies of neurons in SNr (Fig. 2C). These data together suggest that, barring NeuN-immunoreactivity, the gerbil SNr appears to display otherwise normal anatomical characteristics.

NeuN-negative neurons in gerbil SNr receive synaptic input and are electrically excitable

To address the lack of NeuN-immunoreactivity in gerbil SNr more thoroughly, we labeled SNr neurons with biocytin, which was injected intracellularly into individual neurons in acute brain slices during patch-clamp recordings. While most neuron-rich regions within brain slices, as viewed on a fluorescence microscope, were green indicating NeuN-immunoreactivity, SNr was conspicuously devoid of NeuN-labeling bilaterally (Fig. 3A1). This was true of all animals examined (6/6). To visualize double-labeling for NeuN and biocytin at higher magnification, we re-examined slices using laser scanning confocal microscopy. In the example shown in Fig. 3, the biocytin-labeled neuron (red) is located near the SNr-SNc border (Fig. 3A2). High magnification images of the biocytin-filled neuron (Fig. 3A3–A4) confirm it is non-immunoreactive for NeuN (panels in Fig. 3A4 are magnified views of boxed regions in Fig. 3A3). Putative dopaminergic neurons of SNc that are located in the vicinity of the biocytin-filled cell (Fig. 3A3–A5) are, in contrast, NeuN-positive.

To determine whether NeuN-negative cells in gerbil SNr are electrically excitable and to distinguish them from neuron-glial cells, that are also NeuN-negative but electrically non-excitable (i.e., do not fire action potentials [8]), we obtained whole cell voltage- (n = 15 cells) or current-clamp (n = 6 cells) recordings from these cells while labeling them with biocytin. Following fixation, slices were processed for visualization of the biocytin-label and counterstained for NeuN-immunocytochemistry. Fig. 3A6 depicts whole cell voltage-clamp recordings of spontaneous inhibitory post-synaptic currents (IPSCs, outward events at a holding potential of 0 mV) obtained from the SNr neuron of Fig. 3A1–A5. The high frequency of events suggests that these neurons receive substantial synaptic input from GABAergic neurons. The example in Fig. 3B1 shows a biocytin-labeled cell in SNr from which current-clamp recordings shown in Fig 3B2 were obtained. Note that, just as in the previous example (Fig. 3A), the biocytin-filled cell was again non-immunoreactive for NeuN. Resting membrane potential in this cell, and others in this series of experiments, was close to –60 mV, a level that is more depolarized than that reported for glial cells [8,16]. These cells fired action potentials rapidly in response to current injection and displayed little or no spike frequency adaptation (Fig. 3B2). Action potential durations were <1.5 ms and were followed by large amplitude afterhyperpolarizations typical of GABAergic interneurons in SNr [21]. Furthermore, electrophysiological properties of SNr neurons in gerbils [14] were comparable to those in rats [18,21]. These electrophysiological data together with the anatomy of the labeled cells, which is consistent with previous biocytin-labeling studies [14], suggest that these tonically-firing, NeuN-negative cells in SNr are GABAergic neurons.

3. Discussion

This study shows that GABAergic cells in SNr of gerbils, whose neuronal identity was established via electrophysiology, biocytin labeling, histology, and *in situ* hybridization, are not immunoreactive for the neuron-specific marker, NeuN. This deficit appears to be specific to gerbil SNr, because NeuN-immunoreactivity in rat tissue, assayed with the same monoclonal antibody (mAb A60), yielded robust labeling of neurons in SNr. Furthermore, NeuN-labeling in gerbil SNr was lacking in both epileptic and non-epileptic strains and did not show gender-

specificity. Together these data indicate a species-specific difference in the expression of NeuN.

Why might neurons in gerbil SNr be NeuN-negative? A parsimonious explanation is that neurons in the gerbil SNr express little or no NeuN protein. Alternatively, NeuN may be expressed in gerbils, but have reduced antigenicity compared with that of other species. However, the latter possibility is unlikely because lack of NeuN-labeling in gerbils is predominantly restricted to SNr, with robust labeling of other neuron-rich regions within the same sections. NeuN expression levels can be dynamic and reduced in neurons after brain injury induced by metabolic perturbations, without loss of neurons per se [24]. In cultured cerebellar granule cells (the only positively identified neurons in cerebellum that express NeuN), for example, expression of NeuN protein has been correlated with their proliferative capacity and modulation by extrinsic cues. Cerebellar granule cells bearing neurites displayed a higher intensity of NeuN staining on average compared with non-neurite-bearing granule cells [26]. However, it is not known whether expression of NeuN can be regulated in only certain types of neurons and whether it is dependent on location of their origin within the embryonic brain or the stage of maturation [22].

It is unlikely that the observed species-specific difference in NeuN-immunoreactivity is attributable to technical factors such as duration of fixation, storage of the tissue, or incubation of sections at room temperature, which have previously been reported to confound results [22]. Tissue from rats and gerbils was fixed, sectioned, and stored similarly, and processed together. The investigator processing sections was unaware of the species from which it originated. Barring SNr in gerbils, the fact that there was robust NeuN-labeling of brain regions in all examined sections from gerbils and rats suggests that tissue quality was high for both species.

Previous studies have revealed other differences between brains of gerbils and rats. Intra-hippocampal localization of glutamate receptor subtypes in gerbils, when analyzed with a biochemical binding assay, are less region selective than distributions obtained from rats [12]. Immunocytochemical evaluations of gerbil and rat hippocampi revealed mossy cells of the dentate gyrus contain the calcium-binding protein calretinin in gerbils but not in rats [13], and perforant path fibers that project from layer II of entorhinal cortex to molecular layer of dentate gyrus express the calcium-binding protein parvalbumin in gerbils but not in rats [23].

Results of the present study together with previous reports suggest NeuN non-immunoreactive neurons are morphologically diverse and do not appear to share a single neurotransmitter system or any common metabolic feature [22]. Therefore, the answer to the question: why specific cell-types in certain regions of the brain, and now in certain species of animals, are devoid of NeuN-immunoreactivity, might have to await elucidation of NeuN's role in cell function.

Experimental Procedure

Animals

Animals used in this study included 4 Sprague Dawley rats (Harlan, Indianapolis, Indiana) and 12 Mongolian gerbils (*Meriones unguiculatus*). Animals in both groups were 1–2 mo old and of both sexes. Five of the gerbils used were from a line of epileptic gerbils originally derived from a seizure-sensitive strain (WJL/UC) [15], and the remaining were descendants of wild-caught, non-domestic, non-epileptic gerbils [19]. Domestic Mongolian gerbils (which include those procured through commercial vendors) display a high incidence of spontaneous seizure activity [1,15], making it difficult to find a non-epileptic control group [3]. We used recent descendants of wild-caught non-domestic, non-epileptic gerbils to determine whether

differences in NeuN-expression, might be caused by seizure activity. Animals belonging to both genders were considered to determine whether NeuN-expression might be related to previously described sex-specific differences in the topographic segregation and functionality of GABA_A systems in the substantia nigra [25]. All experiments were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Stanford University Institutional Animal Care and Use Committee.

Anatomy

Animals were euthanized by urethane overdose (2 g/kg, i.p.) and perfused through the ascending aorta at 15–30 ml/min with 0.9% NaCl for 2 min and 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min. Brains were postfixed at 4°C overnight, equilibrated in 30% sucrose in 0.1 M PB, and sectioned coronally with a sliding microtome set at 30 µm. Adjacent sections (1-in-6 series, typically comprising of 22–25 sections/animal) were processed for Nissl stain (0.25% thionin), tyrosine hydroxylase-immunoreactivity, or NeuN-immunoreactivity. Sections from both species were processed together.

Immunostaining was performed as described previously [4]. Briefly, endogenous peroxidases were quenched in 1% H₂O₂ for 1 h. Free-floating sections were exposed to blocking solution consisting of 3% goat serum (Vector Laboratories, Burlingame, CA), 2% bovine serum albumin, and 0.3% Triton X-100 in 0.05 M tris buffered saline (TBS) for 1 h. Sections were incubated in rabbit anti-tyrosine hydroxylase serum (1:1200, Chemicon, Temecula, CA) or mouse anti-NeuN serum (1:1000, clone name A60, catalog #MAB377, Chemicon, Temecula, CA) in 1% goat serum, 0.2% bovine serum albumin, and 0.3% Triton X-100 in 0.05 M TBS overnight at 4°C. Sections were exposed to biotinylated goat anti-rabbit serum (1:500, Vector Laboratories, Burlingame, CA) in secondary diluent (2% bovine serum albumin and 0.3% Triton X-100 in 0.05 M TBS) for 2 h and avidin-biotin-horse radish peroxidase complex (1:500, Vector Laboratories) in secondary diluent for 2 h. Color was developed with 0.02% diaminobenzidine, 0.04% NH₄Cl, 0.015% glucose oxidase, and 0.1% β-d-glucose in 0.1 M tris buffer for 12 min.

Adjacent sections from the same gerbils were processed for GAD65 mRNA *in situ* hybridization according to previously described protocols [14]. The GAD65 cDNA (kindly provided by Drs. A. Tobin and N. Tillakaratne, University of California at Los Angeles) was ~2.4 kb and was isolated from a λZapII library from adult rat hippocampus [5]. RNA probes were produced by transcription of GAD65-DNA, using a nonradioactive RNA labeling kit (Roche, Indianapolis, IN). Sections were washed in 10 mM phosphate buffered saline (PBS) and incubated sequentially in 0.01% Triton X-100 in PBS, 0.2 µg/ml proteinase K (Roche) in 50 mM Tris, pH 7.4, 5 mM EDTA (Sigma), and 2 mg/ml glycine (Roche) in PBS. Prior to hybridizing, sections were incubated for 1 hr in a solution containing 50% formamide (Sigma), 750 mM NaCl, 25 mM EDTA, 25 mM piperazine-*N,N'*-bis 2-ethanesulfonic acid (Roche), 0.2% sodium dodecyl sulfate, 250 µg/ml poly A (Roche), and 250 µg/ml salmon sperm DNA (Roche). Sections hybridized for 16–19 hr in a humid chamber at 50°C in a solution consisting of the pre-hybridization solution with the addition of the digoxigenin-labeled RNA probe at a concentration of 2–4 µl/ml, with 100 mM dithiothreitol (Sigma), 4% dextran sulfate (Sigma), and 250 µg/ml tRNA (Roche). After hybridization, sections were subjected to RNase treatment and stringency washes as described previously. Sections were processed for immunodetection of the digoxigenin label with reagents of a nonradioactive nucleic acid detection kit (Roche), mounted on gelatin-coated slides, and covered with Crystalmount (Biomedica, Foster City, CA), PermOUNT, and coverslips.

Electrophysiology

Electrophysiological recordings of gerbil SNr neurons were obtained using previously published techniques [14]. Briefly, coronal slices, 350 μm thick, were cut from brains of gerbils that were deeply anesthetized with urethane (1.5 g/kg, ip) and decapitated. Slices were prepared with a microslicer (VT1000S, Leica) in a chilled (4°C) low- Ca^{2+} , low- Na^{+} “cutting solution” containing (in mM) 230 sucrose, 10 D-glucose, 26 NaHCO_3 , 2.5 KCl, and 1.25 NaH_2PO_4 , 10 MgSO_4 , and 0.5 CaCl_2 , equilibrated with a 95%-5% mixture of O_2 and CO_2 . Slices were stored in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 26 NaHCO_3 , 3 KCl, 1.25 NaH_2PO_4 , 2 MgSO_4 , 2 CaCl_2 , and 10 D- glucose (pH 7.4), first at 32°C for 1 h and subsequently at room temperature before being transferred to a recording chamber. Recordings were obtained at $32 \pm 1^\circ\text{C}$ from neurons in the SNr under Nomarski optics (Nikon, Tokyo, Japan) using a visualized infrared setup (Hamamatsu Photonics, Hamamatsu, Japan). Recording electrodes (1.2–2.0 μm tip diameter, 3–6 M Ω) contained (in mM) 100 potassium gluconate, 40 HEPES, 10 EGTA, 5 for current-clamp recordings MgCl_2 , 2 disodium-ATP, 0.3 sodium-GTP, and 20 biocytin and 120 cesium methanesulfonate, 10 HEPES, 8 NaCl, 2 magnesium-ATP, 0.3 sodium-GTP, 5 QX-314, 0.1 BAPTA, and 20 biocytin for voltage-clamp recordings. Internal solutions were adjusted to a pH of 7.3 with KOH or CsOH and to an osmolarity of 300 mOsm. Postsynaptic currents and potentials were recorded with an Axopatch-1D amplifier and pClamp software (Axon Instruments, Foster City, CA), filtered at 1–2 kHz (10 kHz for current clamp), digitized at 10–20 kHz, and stored digitally.

NeuN–biocytin immunohistochemistry

To visualize biocytin-labeled cells after recording, slices were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) at 4°C for at least 24 h. After fixation, slices were stored in 30% ethylene glycol and 25% glycerol in 50 mM PB at -20°C , before being processed using a whole-mount protocol with counterstaining by NeuN immunocytochemistry. Slices were rinsed in 0.5% Triton X-100 and 0.1 M glycine in 0.1M PB and then placed in a blocking solution containing 0.5% Triton X-100, 2% goat serum (Vector Laboratories, Burlingame, CA), and 2% bovine serum albumin in 0.1M PB for 4 h. Slices were incubated in mouse anti-NeuN serum (1:1000, clone name A60, catalog #MAB377, Chemicon, Temecula, CA) in blocking solution overnight. After a rinsing step, slices were incubated with Alexa Fluor 594 streptavidin (5 $\mu\text{g}/\text{ml}$) and Alexa Fluor 488 goat anti-mouse (10 $\mu\text{g}/\text{ml}$; Invitrogen, Carlsbad, CA) in blocking solution overnight. Slices were rinsed and mounted on slides to which coverslips were applied using Vectashield (Vector Laboratories) before being examined with a confocal microscope (LSM 5 Pascal; Zeiss, Oberkochen, Germany). Filter settings for visualization of biocytin (red channel) and NeuN (green channel) were 543 and 488 nm, respectively.

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Figure 1.

NeuN-immunohistochemistry in rat (**A**) and gerbil (**B**). Substantia nigra *pars reticulata* (SNr) is NeuN-positive in rat but not gerbil. (**A1–B1**) Sections labeled with anti-NeuN mAb A60 show differential staining of SNr in the two species. Note the lack of staining in gerbil SNr compared with intense labeling in other brain stem regions and cerebral hemispheres. (**A2–B2**) Magnified views of boxed regions in **A1–B1** highlighting the conspicuous absence of NeuN-positive neurons in gerbil SNr. (**A3–B3**) Nissl-staining of adjacent sections from the same animals showing numerous neuronal cell bodies in SNr of both rat and gerbil. Scale bar in **A3** applies to all high magnification images (**A2–A3, B2–B3**). SNc = Substantia nigra *pars compacta*.

Figure 2. Gerbil substantia nigra *pars reticulata* (SNr) displays normal neuronal markers. Adjacent sections processed for Nissl stain (**A**), tyrosine hydroxylase (TH)-immunoreactivity (**B**), and GAD65 mRNA *in situ* hybridization (**C**). Higher-powered images in middle and right columns are magnified views of boxed regions in left and middle columns, respectively. SNc = Substantia nigra *pars compacta*.

Figure 3.

Electrophysiological evidence that NeuN-negative cells in gerbil substantia nigra *pars reticulata* (SNr) are neurons. **(A1)** NeuN and biocytin fluorescent labeling in a gerbil brainstem slice viewed through a fluorescence microscope. Note the lack of fluorescence in SNr bilaterally (white arrowheads). **(A2)** Magnified view of boxed regions in right (R) SNr shows a biocytin-labeled cell (red) located near the border of SNr and substantia nigra *pars compacta*. **(A3–A4)** Higher magnification views of boxed regions in **A2** and **A3**. Note that although the biocytin-labeled cell (yellow arrowhead) in SNr is non-immunoreactive for NeuN, others cells in its vicinity including one (red arrowhead) in a different optical plane (Z-axis) are NeuN-positive. **(A5)** Orthogonal views of overlapping cells in **A4** confirm the NeuN-

positive cell (red arrowhead) is at a different depth than the biocytin-filled cell (yellow arrowhead). **(A6)** Whole cell voltage-clamp recordings of spontaneous inhibitory post-synaptic currents (outward events at 0 mV holding potential) obtained from the biocytin-labeled cell shown in **A1–A5**. Bottom traces show time-expanded views of regions indicated by bars under top trace. **(B1)** Confocal image of another NeuN-negative biocytin-filled cell (yellow arrowhead) located in gerbil SNr from which electrophysiological recordings were obtained. **(B2)** Current-clamp recordings from the same cell showing action potential firing when depolarized from rest (resting membrane potential = -62 mV).