

Novel Insights into NeuN: from Neuronal Marker to Splicing Regulator

Wei Duan · Yu-Ping Zhang · Zhi Hou · Chen Huang ·
He Zhu · Chun-Qing Zhang · Qing Yin

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Abstract Neuronal nuclei (NeuN) is a well-recognized “marker” that is detected exclusively in post-mitotic neurons and was initially identified through an immunological screen to produce neuron-specific antibodies. Immunostaining evidence indicates that NeuN is distributed in the nuclei of mature neurons in nearly all parts of the vertebrate nervous system. NeuN is highly conserved among species and is stably

expressed during specific stages of development. Therefore, NeuN has been considered to be a reliable marker of mature neurons for the past two decades. However, this role has been challenged by recent studies indicating that NeuN staining is variable and even absent during certain diseases and specific physiological states. More importantly, despite the widespread use of the anti-NeuN antibody, the natural identity of the NeuN protein remained elusive for 17 years. NeuN was recently eventually identified as an epitope of Rbfox3, which is a novel member of the Rbfox1 family of splicing factors. This identification might provide a novel perspective on NeuN expression during both physiological and pathological conditions. This review summarizes the current progress on the biochemical identity and biological significance of NeuN and recommends caution when applying NeuN immunoreactivity as a definitive marker of mature neurons in certain diseases and specific physiological states.

W. Duan
Department of Neurology, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, People’s Republic of China

Y.-P. Zhang
Department of Pediatrics, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, People’s Republic of China

Z. Hou · C.-Q. Zhang (✉)
Department of Neurosurgery, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, People’s Republic of China
e-mail: cqzhang@tmmu.edu.cn

C. Huang · H. Zhu
Department of Cell and Developmental Biology,
Vanderbilt University, Nashville, TN 37232, USA

H. Zhu
Department of Biological Sciences, Vanderbilt University,
Nashville, TN 37232, USA

H. Zhu
Department of Pharmacology, Vanderbilt University,
Nashville, TN 37232, USA

H. Zhu
The Vanderbilt Kennedy Center for Research on Human
Development, Vanderbilt University, Nashville, TN 37232, USA

Q. Yin (✉)
Department of Rehabilitation and Physical Therapy,
Xinqiao Hospital, Third Military Medical University,
Chongqing 400037, People’s Republic of China
e-mail: qingyin646891@yahoo.com

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Introduction

While preparing monoclonal antibodies against nuclear proteins in mouse brain cells in 1992, Mullen et al. [1] discovered the mAb-A60 monoclonal antibody, which specifically bound a neuron-specific nuclear antigen. This antigen was named neuronal nuclei (NeuN). Immunostaining results using anti-NeuN (mAb-A60) revealed that NeuN is distributed in the nuclei of mature neurons in nearly all parts of the vertebrate nervous system. NeuN is highly conserved among species and is stably expressed during specific stages of development. Therefore, NeuN has been considered to be a reliable marker of mature neurons for the past two decades [2, 3]. The specific

antibody of NeuN, anti-NeuN, is extensively used in many fields, including neuroscience, developmental biology, histology, and stem cell biology, as well as during disease diagnosis [4–6]. For instance, immunocytochemically detectable NeuN protein first appears at developmental stages that correspond to the withdrawal of the neuron from the cell cycle, and/or with the initiation of its terminal differentiation, anti-NeuN is accordingly used for determination neuronal phenotype [5] and identification of neuronal differentiation in diagnostic pathology [7]. In neurological research, the expression level of NeuN has been used to directly evaluate neuronal death or loss, and the reappearance of NeuN-positive cells has become a reliable marker for quantifying therapeutic effects in experimental therapeutic studies [8–10]. Additionally, anti-NeuN has also been widely applied to identify cells as neurons in vitro [11, 12]. However, only a few studies have addressed the biochemical identity and potential function of NeuN [1, 13]. Seventeen years later, NeuN was eventually identified as an epitope of Rbfox3, which is a novel member of the Rbfox1 family of splicing factors [14]. During the past several years, correlative evidence has rapidly accumulated in support of the notion that, rather than being a mere marker of mature neurons, Rbfox3-mediated NeuN immunoreactivity might provide new cues into neuronal biology [3].

Here, we review recent developments elucidating the molecular characteristics and biological significance of NeuN, as a prelude to making better use of this important neuronal marker in neuroscience-related research.

Background of NeuN and Rbfox

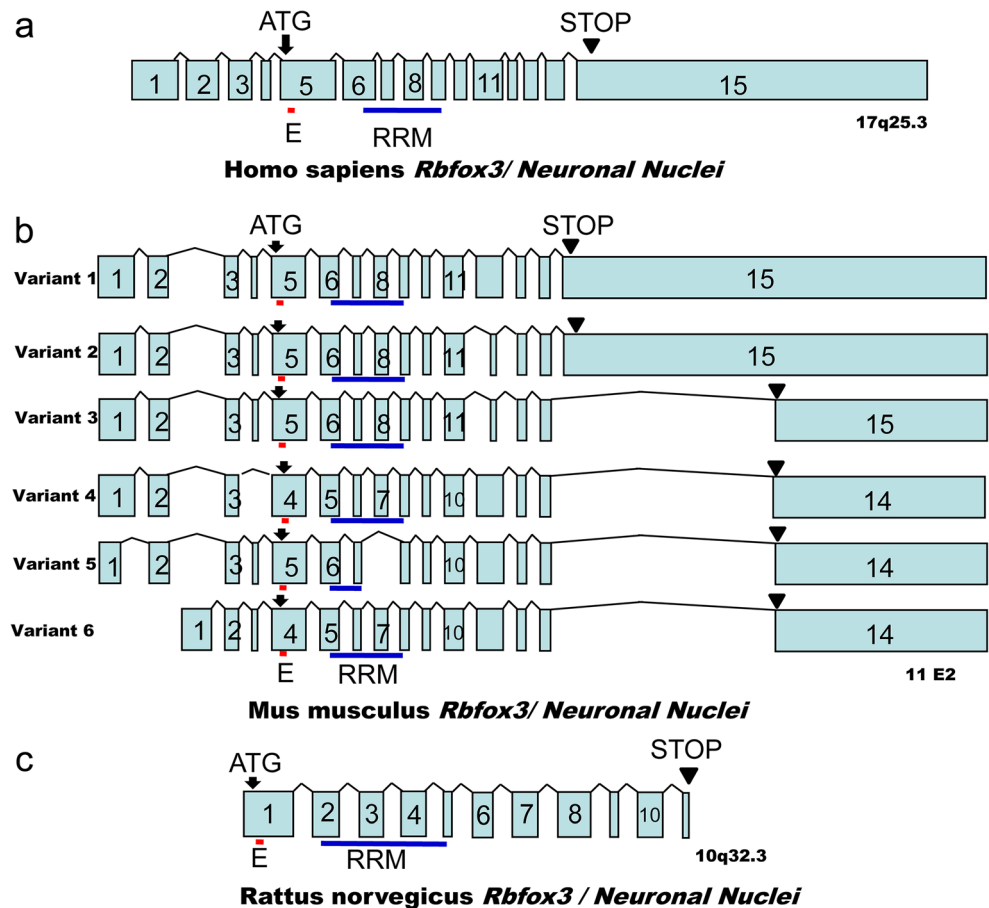
A preliminary study (1992) revealed that NeuN is a soluble nuclear protein that can loosely bind DNA in vitro [1]. Thirteen years later, Lind et al. [13] found that NeuN is a phosphorylated protein. After dephosphorylation treatment, NeuN loses its immunogenicity and cannot be recognized by its specific antibody, indicating that the antibody specificity depends on the phosphorylation status of the NeuN epitope. Recently, a breakthrough study revealed that NeuN is a gene product of *Rbfox-3*, which is a member of the RNA-binding protein Rbfox-1 gene family [14]. This conclusion was based on the following phenomena. (1) Mass spectrometry analyses of the proteins that co-immunoprecipitate with anti-NeuN revealed that the greatest number of protein sequences found in these proteins came from the gene product of *Rbfox-3*. (2) A recombinant Rbfox-3 protein is recognized by anti-NeuN. The epitope recognized by anti-NeuN is found in the N-terminus of Rbfox-3, which includes amino acids 1–106. (3) RNAi-based knockdown of the *Rbfox-3* gene significantly decreases the expression of NeuN. (4) The expression of Rbfox-3 is limited to the nervous system. (5) The patterns of Rbfox-3

and NeuN immunostaining are identical and limited to the nuclei of neurons [14].

Rbfox3 (also known as Fox-3, Fox1 homolog C, Hrnbp3, and *D11Bwg0517e*) is a novel member of the Rbfox1 family of splicing factors. As shown in Fig. 1, in human, *Rbfox3* is a protein-coding gene located on chromosome 17 and comprises 15 exons. In mouse, *Rbfox3* gene is located on chromosome 11, which can generate six variants. Three of the variants are encoded by 15 exons, and the other half variants are encoded by 14 exons. In rat, this gene is located on chromosome 10 and contains 11 exons. *Rbfox3* coding region sequences share extensively: 97 % sequence is identical between mouse and rat, 89 % sequence is identical between mouse and human, and 90 % sequence is identical between rat and human. Rbfox3 protein is also highly conserved across these species, e.g., 98.9 % protein sequence is identical between mouse (isoform I) and rat, and 83.9 % identical between mouse (isoform I) and human as analyzed by Clustal Omega [15]. Recently, the NeuN epitope was mapped to the segment between N-terminal amino acids 6 and 15 (ppaqy-ppppq) [3, 14, 16]. It has been demonstrated that splicing factors are highly enriched in subnuclear structures known as speckles (which vary in shape and size) and that a limited set of proteins are common to the nuclear matrices (NM) of all cell types, whereas other proteins are cell type specific [17, 18]. Interestingly, recent evidence has indicated that Rbfox3/NeuN is an intrinsic component of the NM. Rbfox3/NeuN shuttles between the nucleoplasm and the NM to carry out its role in alternative splicing [19].

Because NeuN binds to DNA and is exclusively expressed in the nucleus, it has been suggested that NeuN might be a transcriptional regulator [1]. Kim's work showing that NeuN is a product of the *Rbfox3* gene further supported this suggestion [14]. In mammals, the Rbfox family includes three members: Rbfox1 (also known as Fox-1 or A2BP1), Rbfox2 (Fox-2, Rbm9, or Fxh), and Rbfox3 (NeuN). The three Rbfox paralogues are a highly conserved family of alternative splicing regulators, all of which contain a single RNA recognition motif (RRM)-type RNA binding domain (RBD) near the center of the protein [20]. Fox-1 and Fox-2 have an identical RBD sequence, and this sequence is only slightly altered in Rbfox3 (4/77 amino acids are substituted) [21]. Rbfox1 is selectively expressed in neurons and in muscle and heart tissue. Rbfox2 is extensively expressed in various tissues, including throughout the embryo, human embryonic cell lines, neurons, and muscle. Rbfox3/NeuN is expressed exclusively in the nervous system. It is well established that Rbfox proteins, especially Rbfox1, regulate a battery of brain- and muscle-specific alternative splicing choices, including exon EIII-B of fibronectin [22], exon N1 of c-src, and exon 33 of the L-type calcium channel Cav1.2 [23], by binding to an RNA penta (hexa) nucleotide (U)GCAUG [24].

Fig. 1 Schematic representation of *Rbfox3/NeuN* gene structure. Exons are indicated as *boxes* with *roman numbers* and approximately drawn to scale; intronic regions are not drawn to scale. **a** The human *Rbfox3/NeuN* gene. **b** The alternative splicing events giving rise to six variants of the *Rbfox3/NeuN* in mice. **c** The rat *Rbfox3/NeuN* gene. (The noncoding regions of rat *Rbfox3/NeuN* gene have not been experimentally identified and not annotated in NCBI Entrez database). The *arrows* indicate translational initiating ATG codon. The *arrowheads* indicate stop codon. *Red* and *blue lines* underneath the scheme indicate the positions of the NeuN epitope (*E*) and the RNA-binding domain (*RRM*). This schematic sketch is based on the data of the NCBI and references [3, 14, 16] (Color figure online)



As a newly identified member of the Rbfox family, the mechanisms of alternative splicing regulation mediated by Rbfox3/NeuN have aroused an increased level of interest over the past few years. Evidence based on the model of neuron-specific alternative splicing of the nonmuscle myosin heavy chain II-B (NMHC II-B) pre-mRNA has demonstrated that Rbfox3 can activate neuron-specific splicing of the cassette exon N30; this activation depends completely on the downstream intronic element UGCAUG[14]. It has been thoroughly demonstrated that alternative splicing of pre-mRNAs is regulated by a cohort of factors in a combinatorial manner [25, 26]. A recent study revealed that the polypyrimidine tract binding protein-associated splicing factor (PSF) interacts with Rbfox-3[27]. The C-terminal region of Rbfox-3 binds directly to the N-terminal region of PSF, but the N-terminal region and the RRM of Fox-3 are not involved in this interaction. PSF enhances the binding of Fox-3 to the target UGCAUG element. Moreover, the presence of PSF enhances the recruitment of Fox-3 in intact cells to the intronic distal downstream enhancer (IDDE) of the NMHC II-B transcript, which contains the UGCAUG elements, to subsequently enhance N30 inclusion. The presence of PSF results in a 30-fold increase in the ability of Fox-3 to bind to the target RNA element in intact cells. These lines of evidence demonstrate that the interaction

between Fox-3 and PSF is an integral part of the mechanism by which Fox proteins regulate the activation of alternative exons via a downstream intronic enhancer[27].

Alternative splicing of pre-mRNA is an important mechanism for the post-transcriptional regulation of gene expression and is generally thought to serve as a major source of functional genomic and proteomic diversity [28]. With advances in human genome research, 70 % of human genes have been discovered to contain alternative splicing subtypes in their mRNA precursors and to participate in transcriptional regulation [29], neurogenesis [30], synapse formation [31], and neurological activities [32], all of which are important in the development and function of the nervous system [33, 34]. More recently, Kim et al. [35] discovered that Rbfox3 knockdown in the developing spinal cord inhibits late neuronal differentiation in post-mitotic neurons without affecting the neuronal fate or subtype specification of progenitor cells located in the ventricular zone (VZ) and intermediate zone of chicken embryos. Moreover, *Numb* pre-mRNA has been identified as a relevant target of Rbfox3-regulated alternative splicing during neuronal development. Knockdown of Rbfox3 reduces exon 12 exclusion in *Numb* pre-mRNA, whereas forced expression of Rbfox3 enhances exon 12 exclusion in a UGCAUG-dependent manner [35].

Strikingly, Rbfox family members can also cross-regulate the alternative splicing of other Rbfox members and auto-regulate themselves [16]. It has been demonstrated that Rbfox3/NeuN promotes the skipping of exon 6 in Rbfox2 and enhances the inclusion of two cryptic exons (exon 5* and exon 6*), leading to increased production of mRNA species that are targeted for nonsense-mediated decay and thereby contributing to the negative regulation of Rbfox2 by Rbfox3. This mechanism of Fox family auto-regulation is likely to be phylogenetically conserved because both of these cryptic exons and their splice sites are highly conserved in both mammals and birds [24]. The complex interplay between Rbfox family members has been further reported in the *Rbfox1* knockout mouse model, in which the loss of *Rbfox1* inhibits the upregulation of Rbfox2 [21].

Expression of Rbfox3/NeuN in Nervous System

Subcellular localization using immunohistochemistry with the anti-NeuN monoclonal antibody has revealed that Rbfox3/NeuN is expressed in the nuclei of mature neurons in nearly every part of the vertebrate nervous system, including neurons in the spinal cord, cerebral cortex, hippocampus, dorsal thalamus, caudate/putamen, and cerebellum [1, 5, 6]. Occasionally, the cytoplasm is also stained but to a lesser extent than the nucleus [36]. Some types of neurons, such as cerebellar granule cells and Dogiel type II neurons, exhibit no staining in the nuclei but are positive for staining in the cytoplasm [37]. According to a previous report, the latter staining might be associated with the penetration of the reagents, the extent of immunoreactivity in the cytoplasm and nucleus, tissue fixation, and the processing of sections [1]. However, recent studies have also shown that these differences in immunoreactivity are primarily due to the distinct subcellular localizations of the various subtypes of NeuN/Rbfox3, such as the 46- and 48-kDa subtypes. The 46-kDa subtype is mainly distributed in the nucleus, whereas the 48-kDa subtype is primarily distributed in the cytoplasm [3]. Additionally, differences in the subcellular localization of NeuN/Rbfox3 might suggest the existence of new cell subtypes. For example, NeuN immunoreactive neurons in cerebellar molecular layer failed to co-label with any cell-type-specific markers, indicating that special cell types or neurons in different physiological states can be distinguished by the differences in NeuN/Rbfox3 expression [38].

Some neurons, including Purkinje cells in the cerebellar cortex, olfactory bulb mitral cells, Cajal-Retzius cells, neurons in the inferior olivary nucleus, dentate nucleus, sympathetic ganglia, retinal photoreceptor cells, and cells in most of the inner nuclear layer, cannot be labeled with NeuN. A recent study also showed that anti-NeuN failed to label a substantial proportion of suprachiasmatic nucleus (SCN) neurons [39].

Moreover, NeuN immunostaining is complete loss in cervical, thoracic, and lumbar segments of the senile rats whereas neuron-specific enolase (NSE) immunoreactivity can be detected in both young and senile animals [40]. The above mentioned neurons vary across several characteristics, including morphology, information integration, and metabolism. Notably, recent evidence has shown that the expression of NeuN varies across species; for example, Kumar et al. [41] found that the neurons in the gerbil substantia nigra pars reticulata do not express NeuN, whereas these neurons in rats strongly express NeuN/Rbfox3.

NeuN Is a Useful Marker of Mature Neurons

NeuN is a sensitive and specific neuronal marker in formalin-fixed and paraffin-embedded tissues. After microwave-mediated antigen retrieval, nearly all nuclei and perikaryons and some proximal processes are strongly positive for NeuN expression, whereas distal axons and the branches of dendrites do not express NeuN. NeuN expression is rare in neuroblasts prior to migration, and it is expressed in neuronal precursors only after migration. In the 19- to 22-week-old cortex, only the deep-layer neuronal precursor cells that will later form layers IV–VI are labeled by NeuN. Although layer II does not exhibit NeuN reactivity, after 24 weeks, most of the neurons exhibit immunopositive reactions. In the cerebellum, pre-migratory external granular layer cells express NeuN at 24 weeks or even earlier. Post-migratory internal granular layer cells do not express NeuN [1, 6]. Because NeuN is primarily expressed in the nucleus, it can be used to accurately and stably label certain neurons with sparse cytoplasm, such as granular cells. Based on its specific expression pattern, recent studies have used NeuN to monitor the neurogenesis of stem cells [42, 43].

More Potential Markers for Neuron

- (1) Microtubule-associated protein (MAP)-2: MAP-2 is a phosphoprotein and critical for neurite extension and branching and for cessation of cell division. MAP-2 is predominantly expressed in the cell body and dendrites of neurons, antibody of MAP-2 is mainly used to label extensively branched neurons from embryonic brain development to adult. [44, 45]. Moreover, MAP-2 expression has also been demonstrated in reactive glia, astrocytomas, and oligodendrogliomas [46, 47].
- (2) Neuron-specific enolase (NSE): NSE was initially considered an acid protease that was specific for neurons and neuroendocrine cells, and its antibody commonly was used to label various types of neurons [48]. However, accumulative studies have shown that NSE is also expressed in the circumventricular organs of the brain and the diffuse neuroendocrine systems of the lung,

intestine, thymus gland, and skin, even in numerous non-neural cells including certain subsets of lymphocytes and smooth muscle cells [49]. Additionally, NSE is a soluble cytoplasmic protein and thus a more difficult than less soluble substances to preserve for anatomical localization studies, and not detected in certain neurons that have sparse cytoplasm [48].

- (3) Neurofilament (NF)-200: NF-200 is a neurofilament with a molecular weight of 200 kDa that distributes in myelinated A-fiber neurons (neurons that generate projection fibers) [50]. NF-200 antibody, RT-97, is a marker of the large and medium-sized, A-fiber neurons typically including large light neurons in dorsal root ganglion and pyramidal neurons in cortex and hippocampus, and rarely used to label other types of neurons [51, 52].
- (4) Synaptophysin: Synaptophysin is a 38-kDa glycoprotein of presynaptic vesicles; its antibody has long been used to identify neurons, certain neuroendocrine cells, and their neoplasms [53]. Synaptophysin is specifically concentrated in axonal terminals than the cytoplasm of neuronal soma; thus, its staining is easily confused with the strong background neuropil. Additionally, Synaptophysin staining is not present in specific types of synapses or their transmitter substances but does concentrate in some nonneural cells (i.e., choroid plexus epithelium) [54]; therefore, Synaptophysin immunoreactivity patterns need to be correlated with other neuronal markers [55].
- (5) SMI-32: SMI-32 antibody recognizes a nonphosphorylated epitope of medium (150 kDa) and heavy (200 kDa) neurofilament proteins, which are believed to be necessary for the structural stability and nutrition transport of large neurons with highly myelinated processes [56]. SMI-32 has been employed to particularly label the dendrites and perikarya of a subset of neocortical pyramidal neurons with subcortical axonal projections [57, 52].

Rbfox3/NeuN Dysregulation in the Neurodevelopmental Diseases

Misregulation or abnormalities in pre-mRNA splicing can result in a number of cellular dysfunctions that manifest as human and animal diseases [58, 59]. Mutations in *Rbfox1* have been implicated in a range of neurodevelopmental diseases, such as idiopathic epilepsy, mental retardation, and attention deficit disorder [60–62]. Several epilepsy candidate genes are downstream targets of Rbfox proteins (GABRB3, GAD2, KCNQ2, SCN8A, FLNA, SLC1A3, DCX, SLC12A5, SV2B, and SYN1), and regulation mechanisms of the expression and splicing of these genes by Rbfox members have been

demonstrated [63, 64]. Interestingly, a recent study demonstrated that a 43-kb deletion spanning exon 3 and a nonsense mutation (p.Y287*) in *Rbfox3* are found in patients with Rolandic epilepsy (RE), which is one of the most common epilepsy syndromes among children; these findings indicate that exon deletions and truncating mutations of *Rbfox3/NeuN* contribute to the genetic variance of idiopathic epilepsy syndromes [65].

Apparently balanced chromosomal rearrangements (ABCs) have been strongly associated with human neurodevelopmental diseases [66]. Utami et al. [67] more recently mapped the ABCR breakpoints in patients with neurodevelopmental disorders. In this study, *Rbfox3/NeuN* gene was identified within the ABCR breakpoint regions in patients with developmental delay and speech disorders, and an enrichment of DNA copy number variations was detected in *Rbfox3/NeuN* gene, suggesting a putative role for *Rbfox3/NeuN* in the pathogenesis of the neurodevelopmental diseases [68].

The Application of Rbfox3/NeuN as a Controversial Marker in Neurological/Neuroscience Research

Stroke, PD, SUPD, HIV, and TSC

Studies of changes in the morphologies, functions, numbers, and spatial distributions of neurons during stroke are important for identifying disease mechanisms, for developing routes of prevention and therapy, and for investigating the plasticity of nerve tissues. Because NeuN is a stable and reliable marker of mature neurons, it has been widely used in stroke research. On the one hand, the expression status of NeuN has been used to directly evaluate neuronal death or loss [8–10]. On the other hand, the reappearance of NeuN-positive cells has become a reliable marker for quantifying therapeutic effects in experimental therapeutic studies [69, 70].

Notably, the results of recent studies of NeuN in the stroke field remain controversial. Some studies of stroke have revealed that NeuN-positive cells are indeed decreased to a greater extent in the disease-related foci than in normal regions, and this phenomenon has been suggested to be caused by neuronal death or loss [9, 10]. However, Unal-Cevik et al. [71] found that the loss of NeuN staining in the disease foci in stroke is not due to neuronal death but rather to a decrease in NeuN protein expression or a loss of NeuN antigenicity. This study revealed that in MCAO rats, NeuN-positive neurons are decreased by 27 % in the penumbra and 62 % in the ischemic core area. However, hematoxylin and eosin staining did not reveal significant neuronal loss. Labeling with the neuron-specific marker caspase-3p20 after ischemia has revealed that the observed caspase-3p20-positive neurons are NeuN-negative, and labeling with the nuclear dye Hoechst 33258 has also

suggested that there are intact neuronal nuclei in NeuN-negative regions. These results indicate that NeuN-negative staining does not necessarily represent neuronal death or loss and raise questions regarding previous results. The loss or reappearance of NeuN immunoreactivity cannot be strictly interpreted to indicate neuronal loss or reappearance. Accordingly, it might be necessary to re-examine previous studies that used positive NeuN immunoreactivity for neuronal counting and re-evaluate the conclusions of those studies.

The loss of dopaminergic neurons in the substantia nigra is an important pathological feature of Parkinson's disease (PD) [72]. Studies in several classic animal neurotoxicity models of PD (e.g., 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, paraquat, and rotenone models) have all reported neuronal loss in the substantia nigra [73–75]. The accurate counting of dopaminergic neurons is an important method for evaluating PD models and is also an important indicator of the neuroprotective effects of treatment [74, 76]. Because NeuN is expressed in the nuclei of most neurons, it is extensively used to observe the loss of markers of specific cell phenotypes and to reflect the loss of and damage to specific cell types in PD and its disease models. The quantification of neurons is an important method of evaluating neurodegenerative models and neuroprotective functions. NeuN has been used extensively to study neuronal loss in the substantia nigra [77–79], but this method has recently been challenged.

One recent study by Cannon et al. [80] showed that the degree of NeuN staining in dopaminergic neurons varies widely from undetectable to strong. The expression of NeuN in neurons in the substantia nigra of the rats exhibits significant variation across various physiological conditions. Many dopaminergic neurons either do not express NeuN or express only low levels of NeuN. Additionally, the expression patterns of NeuN range from simple cytoplasmic staining to whole-cell staining. These phenomena have been observed in all experimental animals and are not associated with fixation methods. At the subcellular level, the expression patterns of NeuN also exhibited large variations. For example, many neurons in the dorsal and ventral substantia nigra exhibit NeuN expression in the nucleus and cytoplasm. The expression level of NeuN in nondopaminergic neurons in the ventral midbrain is significantly higher than that in dopaminergic neurons, and NeuN exhibits nuclear and cytoplasmic staining in these peripheral neurons of the substantia nigra. These results suggest that NeuN is not a reliable marker of dopamine neurons in the substantia nigra [80]. Therefore, it is necessary to develop more accurate methods to quantify all neurons in the substantia nigra and to judge the characteristic loss of dopaminergic neurons.

A recent study evaluated the immunoexpression of NeuN in a group of victims of sudden unexplained perinatal death, including cases of sudden intrauterine unexplained death syndrome (SIUDS) and sudden infant death syndrome (SIDS).

The findings demonstrated that, in control cases, the major part of the neurons (24/30) exhibited strong NeuN immunostaining in nearly the entire neuronal cell populations (the pontine nuclei served as the region of observation). However, NeuN staining was not detected in 63 % of SIDS and 69 % of the SIUDS cases, and decreased NeuN staining intensities were observed in 21 % of the SIDS and 6 % of the SIUDS cases. Notably, the neuropathological results revealed no changes in the numbers or morphologies of neurons in this area compared to the controls. Furthermore, both TUNEL and caspase 3 staining failed to reveal apoptotic features in brain samples from the sudden unexplained perinatal death cases. Thus, the loss of NeuN staining in neurons with an otherwise healthy appearance in sudden unexplained perinatal death does not necessarily indicate neuronal loss or an ongoing process of neuronal death but might simply indicate a decrease in NeuN protein synthesis or change in NeuN antigenicity [81].

Human immunodeficiency virus type I (HIV-1)-associated neurocognitive disorders (HANDs) are a specific neuronal dysfunction that arises as a sequela of HIV-1 infection in macrophages and microglia in the brain. Findings have indicated that 33–60 % of HIV-infected patients suffer cognitive impairment [82]. HAND is categorized into three subgroups: HIV-associated dementia (HAD), minor neurocognitive disorder (MND), and asymptomatic cognitive impairment (ANI) [83]. The typical pathological features of HAD include activated macrophages and microglia; extensive neuronal apoptosis; and astrogliosis in multiple brain regions, including the frontal cortex, hippocampus, cerebellum, and striatum. In MND and ANI, synaptodendritic injury rather than neuronal loss is the dominant feature [84, 85]. Lucas et al. [86] used anti-NeuN to observe the neuron distributions in HAND brain tissues acquired from autopsy. Intriguingly, their results revealed that NeuN staining was pronounced in the cell bodies and axons and not prominently nuclear as previously reported. Moreover, neurons with both nuclear and cytoplasmic anti-NeuN reactivity were also readily detected. This distribution pattern contrasts with the widely reported nuclear localization of anti-NeuN.

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutations in *TSC1* or *TSC2*. Cortical tubers are the most prominent brain lesions in TSC and represent a well-recognized cause of epilepsy. The abnormal cells known as dysplastic neurons (DNs) and giant cells (GCs) are typical pathological hallmarks in cortical tubers of TSC [87]. Recently, Zhang et al. [88] found that vascular endothelial growth factor-C (VEGF-C), a new neurotrophic factor, is highly expressed in DNs and GCs, and immunofluorescence staining revealed the co-localization of VEGF-C immunostaining with the neuronal marker NF-200 in DNs and GCs. However, none of the VEGF-C-positive DNs and GCs co-expresses the NeuN, suggesting a decrease in NeuN protein synthesis or disappearance of NeuN antigenicity in TSC.

Axotomy, Chemical, and Physical Stress

Axonal injury and axotomy can cause serious neuronal shrinkage and death [89]. McPhail et al. [90] demonstrated that the use of NeuN as a marker for neuronal counting following axonal injury might yield incorrect conclusions. These authors established a peripheral nerve axotomy model (via facial nerve resection) and a central nerve axotomy model (via rubrospinal tract resection in the neck plane) and evaluated the expression of NeuN using the anti-NeuN antibody. The results revealed that 3 days after peripheral nerve injury, NeuN immunoreactivity in the facial motor neurons was completely lost but began to return within 7 days of the injury and returned to the uninjured expression level by 28 days. Moreover, after axotomy in the central nervous system, the expression of NeuN declines little in rubrospinal neurons. The above results indicate that the changes in NeuN immunoreactivity that follow axotomy differ across types of neurons. Therefore, NeuN might be an inappropriate marker for neuronal counting following axotomy [90].

Chemical and physical stresses can affect the survival status of neurons [91]. Using hemalum-phloxine and Fluoro-Jade B staining, Collombet et al. [92] found that approximately 49 % of rat hippocampal neurons are damaged after soman (a neurotoxin) poisoning at 1.2 times the LD₅₀ dose. NeuN staining of the same sections did not reveal NeuN-positive cells, whereas Western blot revealed the expression of NeuN in hippocampal regions. These results indicate that the loss of NeuN staining was due to the disappearance of protein immunogenicity rather than a decrease in the protein expression level.

Another study showed that after 17-Gy whole-brain radiation, the number of NeuN-positive hippocampal neurons significantly decreases, whereas the immunoreactivity for other neuronal markers, i.e., calbindin D28k and synaptophysin1, is not significantly decreased. Additionally, the total numbers of granule and pyramidal cells also remain unchanged. Therefore, irradiation might lead to a temporary loss of NeuN protein expression in the mouse hippocampus. However, these changes do not necessarily indicate neuronal loss, suggesting the need for caution regarding the use of

Table 1 Summary of the applications of Rbfox3/NeuN as a controversial marker in neurological/neuroscience research

Disease/State	NeuN Immunoreactivity	Main finding	References
Stroke	Negative	The loss of Rbfox3/NeuN-positive cells does not indicate the loss of neurons; it could be due to the loss of Rbfox3/NeuN immunogenicity	Unal-Cevik et al. [71], 2004
Parkinson's disease	Negative	The staining of Rbfox3/NeuN in dopaminergic neurons varies widely from undetectable to strong under physiological conditions, and many dopaminergic neurons either did not express Rbfox3/NeuN or expressed only low levels	Cannon et al. [80], 2009
Sudden unexplained perinatal death	Negative	Rbfox3/NeuN staining was not detected in the majority of sudden unexplained perinatal death cases. However, there were no changes in the numbers or morphologies of neurons compared with those of the same brain region in controls	Lavezzi et al. [81], 2013
Tuberous sclerosis complex	Negative	Dysplastic neurons/giant cells expressing VEGF-C co-label with the neuronal marker NF-200 rather than NeuN, suggesting a decrease in NeuN protein synthesis or disappearance of NeuN antigenicity in TSC	Zhang et al.[88], 2012
Neurotoxin: soman	Negative	After soman poisoning, the loss of Rbfox3/NeuN staining was due to the disappearance of protein immunogenicity rather than a reduction in the protein expression level	Collombet et al. [92], 2006
Radiation	Negative	Irradiation leads to a temporary loss of Rbfox3/NeuN protein expression in the mouse hippocampus. This change does not necessarily indicate the loss of neurons	Wu et al. [93], 2010
Senile spinal cord	Negative	Rbfox3/NeuN immunostaining is complete loss in cervical, thoracic, and lumbar segments of the senile rats	Portiansky et al.[40], 2006
In vitro culture conditions	Positive	The anti-NeuN antibody can recognize the nuclei of astrocytes cultured from fetal and adult humans, newborn rats, and embryonic mouse brain tissues	Darlington et al. [94], 2008
HIV-associated neurocognitive disorders (HANDs)	Abnormal distribution	In HAND brain tissue from autopsy, Rbfox3/NeuN staining was pronounced in the cell bodies and axons but not prominent in the nuclei, as reported previously	Lucas [86], 2014
Axotomy	Varied	After axotomy, changes in Rbfox3/NeuN expression vary according to the course of disease and in separate neuronal populations, indicating that care must be taken when addressing cell survival based on Rbfox3/NeuN staining alone	McPhail et al. [90], 2004

phenotypic markers, such as NeuN, to estimate changes in neuronal numbers after irradiation [93].

Rbfox3/NeuN in Cell Culture

Many studies have used NeuN as a marker for post-mitotic neurons under in vitro culture conditions [11, 12]. It is widely believed that NeuN expression has not been observed in glial cells under any circumstances [1]. However, Darlington et al. [94] recently found that the anti-NeuN antibody can recognize the nuclei of astrocytes cultured from fetal and adult humans, newborn rats, and embryonic mouse brain tissue. Although these NeuN-positive astrocytes were proliferating, no evidence of neurogenesis was detected. These authors also obtained positive NeuN immunostaining in the nonneuronal fibroblast cell line 3T3. In sections of P10 rat brains, the expression of NeuN is limited to neurons and absent from astrocytes. These results are consistent with the application of NeuN as a selective marker of mature neurons in vivo but indicate that the expression range of NeuN is more widespread in vitro than originally thought and is limited neither to neurons nor to post-mitotic cells. Therefore, incorrect conclusions might be obtained when applying NeuN as a marker of mature neurons during in vitro experiments. Studies utilizing NeuN as a definitive marker of post-mitotic neurons in vitro should be confirmed by staining for additional neuronal and glial cell markers.

Conclusions

Since its discovery, NeuN has become one of the most recognized neuronal markers due to its broad distribution patterns and specific localization in the nervous system. However, a number of recent studies have argued that NeuN immunoreactivity might not be a reliable marker of neuronal survival or neuron numbers during certain diseases and specific physiological states (Table 1). Therefore, studies utilizing NeuN immunoreactivity as a definitive marker of mature neurons in these conditions should be interpreted with caution.

The recent identification of NeuN as Rbfox3 has provided a novel perspective on NeuN immunoreactivity and its interpretation; i.e., the perception of NeuN as only a simple neuronal marker might be too restrictive. Lost/decreased production of Rbfox3/NeuN and the translocation of Rbfox3/NeuN from the nucleus to the cytoplasm might lead to the downregulation of the alternative splicing of the RNA of its target genes and thereby change the complement of neuronal-specific gene expression. Future studies will seek to identify the genes regulated by Rbfox3, which might be important in neuronal survival and homeostasis. Moreover, the identification of NeuN as an alternative splicing factor provides one more fascinating example of an RNA-binding protein that is

strictly neuron specific, such as the Nova [95] and HuD [96] proteins.

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