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NeuN/Fox-3 is an intrinsic component of the neuronal nuclear matrix

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ABSTRACT

NeuN is an antigen detected in the nucleus of neurons in a wide range of vertebrates and so it is widely used as a tool for detecting neuronal cells. NeuN has been recently identified as Fox-3, a new member of the Fox-1 gene family of splicing factors. The predominant localization of NeuN/Fox-3 to neuronal nuclei and its role in splicing pose the question of the nuclear compartmentalization of such a protein. Here we provide evidence that NeuN/Fox-3 is an intrinsic component of the neuronal nuclear matrix and a reliable marker of nuclear speckles in neurons.

Structured summary:
MINT-7890176: *Fox-3* (uniprotkb:B7ZC13) and *Splicing factor SC35* (uniprotkb:Q6PDU1) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

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1. Introduction

NeuN (neuronal nuclei) is an antigen that is consistently detected by the corresponding monoclonal antibody anti-NeuN in the nucleus of most types of neurons [1,2] in a wide range of vertebrates including mammals, birds and amphibians. Therefore, anti-NeuN is widely used as a tool for detecting neuronal cells from the central and peripheral nervous systems. Recently it has been clarified the actual identity of the NeuN protein that was identified as Fox-3, a new member of the Fox-1 gene family of splicing factors that functions as regulator of splicing [3]. The predominant localization of NeuN/Fox-3 to neuronal nuclei and its role in splicing of primary transcripts pose the question of the nuclear compartmentalization of such a protein. The nuclear matrix (NM) has been operationally defined as the nuclear substructure that results from extracting cells in presence of high-salt, non-ionic detergents and DNase [4–6]. The specific composition of the NM is still a matter of debate as some four hundred proteins have been associated with this substructure yet about 50% of such proteins appear to be components of the internal NM whereas others exhibit a cell-cycle-dependent association with the substructure [7]. However, at least in mammals, there is evidence for a limited set of proteins common to the NM of all cell types [8] whereas others are cell-type-specific [9]. There is varied evidence that fundamental processes of nuclear physiology, such as replication, transcription and splicing of primary transcripts, occur at macromolecular complexes organized upon the NM [10–12]. Moreover, numerous observations indicate that the so-called nuclear speckles enriched in splicing factors for pre-messenger RNA interact with the NM [13–16]. Here we provide evidence that NeuN/Fox-3 is an intrinsic component of the neuronal NM and a reliable marker of nuclear speckles in neurons.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–250 g were used for isolation of rat neuronal nuclei. All animals were used in accordance with the official Mexican norm for production, care and use of laboratory animals (**NOM**-062-ZOO-1999).

2.2. Neuronal nuclei isolation

Neuronal nuclei from cerebral cortex of young-adult rats were isolated as described [17] with slight modifications. The cerebral cortex was dissected and then homogenized with 1 ml of 2.0 M sucrose (1 mM $MgCl_2$ + 0.25 mM PMSF). The homogenate was transferred to a tube containing 5 ml of 2.0 M sucrose and spun at 4 °C for 60 min at 49 000×g. The pellet was washed with 10 ml of 0.32 M sucrose (1 mM $MgCl_2$ + 0.25 mM PMSF) and spun for 5 min at 1500×g. The final pellet was resuspended in 1 ml of 0.32 M sucrose.

2.3. Preparation of nuclear substructures

1×10^6 nuclei from cerebral cortex cells were suspended in 0.5 ml of TM-2 buffer (2 mM $MgCl_2$; 0.5 mM PMSF; 10 mM Tris

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pH 7.2). From this suspension 25 μl were laid on a microscope slide and allowed to dry for 1 h at room temperature. Nuclear substructures were obtained as previously described [18]. Briefly, for nucleoid preparation in situ the dried sample was treated with 500 μl of lysis buffer (2.6 M NaCl; 1.3 mM EDTA; 2.6 mM Tris; 0.6% Triton X-100; pH 8.0) for 10 min at 4 °C, and washed in PBS without Ca^{2+} and Mg^{2+} (PBS-A). For obtaining the DNA-depleted NM the dried nucleoids were treated in situ with 500 μl of DNase I (7 U/ml) at 37 °C for 3 h under continuous shaking. The slides were washed with PBS-A (5 min) and next with ultra-pure water at room temperature. For obtaining the DNA–RNA-depleted NM in situ the samples were digested with 300 μl of RNase A (at 200 μg/ml; 86 U kunitz/mg) for 1 h at 37 °C and then washed with PBS-A.

2.4. Immunofluorescence

For immunofluorescence all kind of samples were fixed for 15 min with 4% paraformaldehyde in 0.1 M NaH_2PO_4 + 0.1 M Na_2HPO_4, pH 7.4. Sample slides were treated either with monoclonal mouse anti-NeuN (1:500, Chemicon), rabbit polyclonal anti-Lamin B1 (1:2000, Abcam), goat polyclonal anti-SC35-D-18 (1:100, Santa Cruz Biotech.), goat polyclonal anti-SRm160-K-15 (1:100, Santa Cruz Biotech.) in PBS-A with 10 μl of 10% milk and 1.5 μl Tween 20, in a humidified chamber 4 h at 4 °C. The anti-NeuN treated samples were further treated with Alexa fluor 568 (red, 1:2000) in PBS-A in a humidified chamber for 1 h at 4 °C. Anti-Lamin B1 treated samples were further treated with biotinylated goat anti-rabbit IgG (1:500, Vector) in PBS-A with 10 μl of 10% milk in a humidified chamber for 1 h at 4 °C and further treated with fluorescein-avidin (1:500, Vector) for 30 min at 4 °C. The anti-SC35 and anti-SRm160 treated samples were further treated with biotinylated horse anti-goat IgG (1:1000, Vector) in PBS-A with 10 μl of 10% milk in a humidified chamber for 1 h at 4 °C and then further treated with fluorescein-avidin (1:1000, Vector) for 30 min at 4 °C. The slides were mounted with VectaShield. Samples were observed in an Olympus BX60 fluorescence microscope. Analysis of co-localization of the specific fluorescent signals was done using the Image-Pro-Plus Analysis System.

2.5. Western blot

Total protein extraction and quantification was done by standard procedures. Protein samples were subjected to standard PAGE in 4–10% discontinuous gels. The proteins were electro-transferred to a nitrocellulose membrane Hybond-ECL (GE Healthcare). The membrane was incubated in TBST buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween, pH 8.0) with 10% milk for 2 h at room temperature. Next the membrane was treated with mouse anti-NeuN (1:5000) or mouse anti-Actin (1:40 000; Millipore) in TBST buffer with 5% milk for 1 h. After three washes with TBST the membrane was incubated with biotinylated horse anti-mouse, rat-adsorbed, IgG (1:3000, Vector) in TBST with 5% milk for 1 h. Then treated with the pre-mixed A + B reagents from VectaStain elite ABC kit (Vector) for 30 min. The signal was developed with Amersham ECL reagent (GE Healthcare) for 2 min and captured on ECL Hyperfilm.

3. Results

3.1. Differential identification of nuclei and nucleoids from neurons

In nuclei and nucleoid preparations obtained from cells of the rat cerebral cortex the neuron-specific nuclei and nucleoids were detected by indirect immunofluorescence using a mAb against the neuron-specific nuclear protein NeuN. The percentage of neuron-specific nuclei and nucleoids was estimated by counting positive-stained nuclei/nucleoids against non-stained. In fresh preparations of nuclei the contamination by NeuN-negative nuclei (glial-cell nuclei) was <27%. However, after extracting the rather mixed nuclei for obtaining nucleoids, the contamination by NeuN-negative nucleoids fell to <8%, indicating that most glial-cell nuclei were unable to withstand the nucleoid extraction procedure. Hence the resulting nucleoid preparations were highly enriched (>92%) in neuron-specific nucleoids and so it is possible to use the isolated nuclei from cerebral cortex for obtaining NM preparations that basically consist of neuron-derived substructures.

3.2. Characterization of nuclear substructures from rat neuronal nuclei

Nuclei (Fig. 1A) were extracted with high-salt and non-ionic detergent for obtaining nuclear substructures known as nucleoids (Fig. 1D) in which the naked nuclear DNA, organized in supercoiled loops, remains attached to the NM [18,19]. However, treatment of nucleoids with the DNA-intercalating agent ethidium bromide (EB) causes unwinding of the supercoiled DNA loops [18,20], since the EB acts as a molecular lever leading to the formation of a fluorescent DNA halo that surrounds the NM (Fig. 1E and F). Treatment of nucleoids with DNase I leads to disappearance of the fluorescent DNA halo remaining a faint fluorescence resulting from the staining of nucleoli and internal ribonucleoprotein within the boundary of the NM (Fig. 1H and I). Indeed, it is known that heterogeneous nuclear ribonucleoprotein (hnRNP) constitutes a significant component of the NM internal fibrogranular network [4,21]. Further treatment of DNA-depleted NM preparations with RNase A leads to disappearance of any residual fluorescence after exposure to EB (Fig. 1K and L).

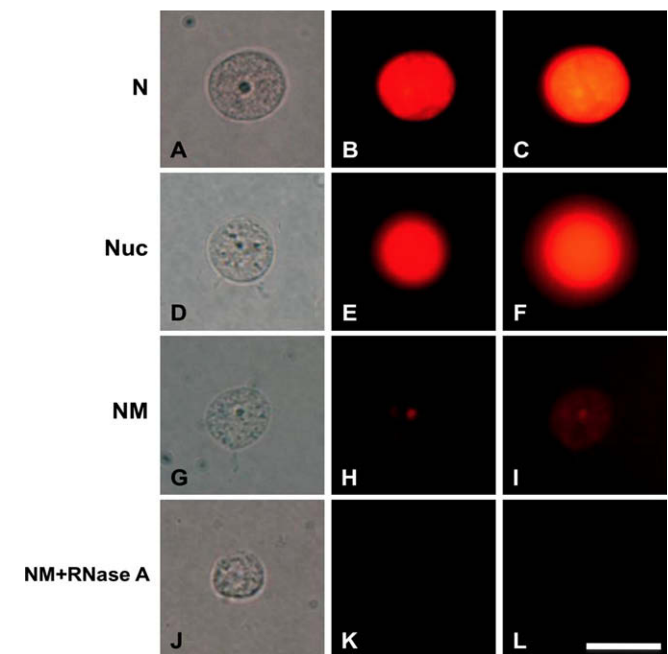

Fig. 1. Microscopic characterization of neuronal nuclear substructures. Representative phase-contrast micrographs of: neuronal nuclei (A), nucleoids (D), DNA-depleted NM (G) and DNA–RNA-depleted NM (J). The samples were treated with ethidium bromide (EB) at a final concentration 80 μg/ml for staining DNA/RNA. Fluorescent micrographs were obtained after differential sample-exposures: B and E = 10 ms; C, F, H and K = 50 ms; I and L = 200 ms. Notice the EB-induced DNA halo surrounding the NM in the nucleoid preparation (F). The faint internal fluorescence in the DNA-depleted NM sample is from the residual nucleoli and the internal fibrogranular RNP network (H and I). EB-fluorescence is not detectable in the DNA–RNA-depleted NM even after a 200 ms exposure (K and L). Bar = 10 μm.

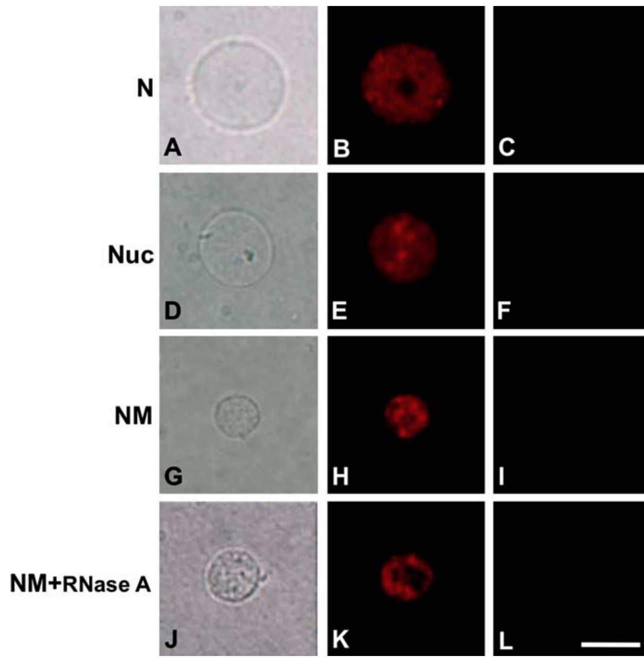

Fig. 2. Detection of NeuN/Fox-3 in neuronal nuclear substructures. Phase-contrast micrographs of: nuclei (A), nucleoids (D), DNA-depleted NM (G), DNA–RNA-depleted NM (J). NeuN-specific indirect immunofluorescence was detected at differential sample-exposures: B and E = 100 ms; H and K = 50 ms. Notice that sample exposure had to be reduced in the NM preparations (H and K) so as to avoid saturation, indicating that removal of both the nuclear membrane and nuclear DNA significantly reduced the quenching of the fluorescent signal resulting from NM-bound NeuN. The corresponding negative controls in which the primary anti-NeuN mAb was excluded (C, F, I and L) confirm the immunofluorescence specificity. Bar = 10 μm.

3.3. NeuN immunofluorescence

Neuronal nuclei and the three different kinds of nuclear substructures: nucleoids, DNA-depleted NM and DNA–RNA-depleted NM, were subjected to indirect immunofluorescence with anti-NeuN. The results show that NeuN is present in the nucleoplasm since there is a reduced immuno-staining for NeuN in neuronal nucleoids when compared with that in the original neuronal nuclei (Fig. 2B and E). However, nucleoids contain about 10% of the total nuclear protein [22] and yet there is a significant immuno-staining for NeuN in such substructures. The staining is shared between a few large speckles and a diffuse immuno-staining within the boundary of the nucleoid, suggesting that some portion of NeuN may interact with the naked loop DNA and/or the internal network of ribonucleoprotein (Fig. 2E). When DNA is removed by treatment with DNase I the resulting NM shows significant staining for NeuN that is concentrated in a few large speckles although there it remains some diffuse staining in the internal NM (Fig. 2H). However, after removing the internal ribonucleoprotein network by treatment with RNase A, the sharp NeuN staining concentrates in speckles, which in some preparations appear to be peripherally-located and somehow fused to each other (Fig. 2K).

Lamin B provides structural support to the nuclear membrane and is a major component of the peripheral NM [23] defining a smooth boundary for the whole neuronal nucleus and the neuronal NM preparations (Fig. 3). Comparison of the immunofluorescent staining patterns for both NeuN and lamin B in the neuronal NM preparations, confirms that a significant portion of NeuN is concentrated in speckles that are preferentially located within the periphery of the NM (Fig. 3B and F). The serine/arginine-rich splicing factor SC35 and the serine/arginine-rich splicing co-activator SRm160 are two reliable markers of nuclear speckles also known to be associated with the NM [15,24], the immunofluorescent

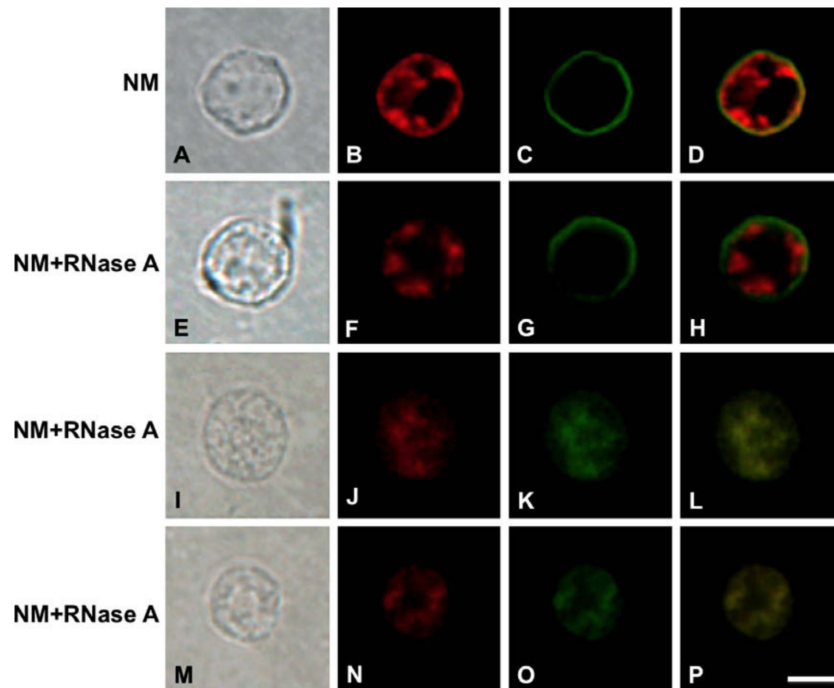

Fig. 3. Comparative immunofluorescent patterns for NeuN/Fox-3, lamin B1, SC35 and SRm160. (NM) DNA-depleted NM (A) and (NM + RNase A) DNA–RNA-depleted NM (E, I and M) were immunostained by specific indirect immunofluorescence for NeuN (B, F, J and N), lamin B1 (C and G), splicing factor SC35 (K) and co-activator of splicing SRm160 (O). Notice the significant presence of NeuN/Fox-3 in peripherally-located NM-bound speckles (B and F) while lamin B1 immunofluorescence is restricted to a smooth stained rim that do not co-localizes with NeuN/Fox-3 (D and H). The immuno-staining pattern for NeuN/Fox-3 in DNA–RNA-depleted neuronal NM matches almost perfectly (co-localization coefficient >0.99) with those for SC35 (L) and SRm160 (P). Bar = 5 μm.

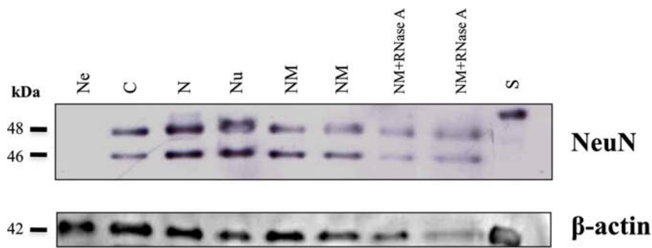

Fig. 4. Detection of NeuN/Fox-3 by Western blot. Similar quantities (15 μg) of total extracted proteins from rat peripheral sciatic nerve (Ne), cerebral cortex (C), neuronal nuclei (N), neuronal nucleoids (Nu), neuronal DNA-depleted NM (MN), neuronal DNA–RNA-depleted NM (NM + RNase A) and spleen (S), were subjected to detection of NeuN/Fox-3 with specific anti-NeuN mAb. Notice the presence of both NeuN/Fox-3 isoforms (46–48 kDa) in all samples except peripheral nerve and spleen (negative controls). Both NeuN/Fox-3 isoforms are readily detected in the ultimate DNA–RNA-depleted NM. β-Actin (42 kDa) was detected in all lanes as control for whole protein loading.

staining patterns for NeuN and for each of such markers of speckles match almost perfectly (co-localization coefficient >0.99) in neuronal NM preparations (Fig. 3L and P).

3.4. NeuN/Fox-3 protein in neuronal nuclear substructures

Western blot analysis of the different nuclear substructure preparations (Fig. 4) confirms that some portion of NeuN is present in the nucleoplasm and that a further portion may be associated to the internal ribonucleoprotein network as judged by comparing the corresponding NeuN signals in nuclei, nucleoid and NM preparations. However, a sizeable portion of NeuN remains in DNA–RNA-depleted NM preparations despite the fact that such preparations are known to contain only some 10% of total nuclear protein, less than 1% total DNA and less than 5% total RNA [22], strongly suggesting that NeuN is an intrinsic component of the NM of neurons.

4. Discussion

The antigen NeuN recently identified as the Fox-3 splicing factor is a protein specifically expressed in most types of neurons, thus it is a reliable marker of neuronal identity. Fox-3 is a regulator of splicing that activates neural cell-specific splicing [3]. Splicing factors are known to be enriched in subnuclear structures known as speckles that vary in size and shape. Such speckles show a dynamic behavior since both their protein and ribonucleoprotein components cycle continuously between the speckles and other nuclear locations that include active transcription sites [25]. Yet the speckles are known to interact directly with the NM [4,15,16]. The observed intranuclear compartmentalization of NeuN/Fox-3 is consistent with its role as a splicing factor thus being present in the nucleoplasm, where it may interact with hnRNP, as well as being concentrated in discrete speckles bound to the NM (Figs. 2–4). Thus, NeuN/Fox-3 is likely to be shuttling between the nucleoplasm and the NM. Yet there is always a sizeable portion of the splicing factor that is consistently bound to the ultimate NM fraction of neurons, despite the rather harsh procedure for isolating such a substructure that removes some 90% of the original nuclear protein and almost all DNA and RNA [18,22]. Therefore, NeuN/Fox-3 may be regarded as an intrinsic component of the neuronal NM. Topoisomerase II alpha is also an intrinsic NM protein that nevertheless shuttles continuously between the NM and the nucleoplasm. This enzyme has been consistently isolated in the ultimate NM fraction of most cell types [26,27] yet it exchanges continuously with the soluble pool of nuclear proteins [28]. Such a behavior is not in conflict with the current concept of the NM as both a dynamic structure and a nuclear compartment in which most of the proteins are not firmly restrained but continuously shuttle between the NM and the nucleoplasm [6]. Hence the NM is a structured milieu in which concentrated nuclear proteins statistically increase their probabilities for interaction, thus facilitating the formation of macromolecular complexes organized on the NM that participate in the fundamental processes of nuclear physiology such as replication, transcription and splicing. Indeed, SRm160 a co-activator of splicing present in nuclear speckles contains structural motifs that target the protein to NM sites at splicing speckled domains [15]. Such a protein forms a stable complex with a 300 kDa NM-antigen [29]. Thus it is remarkable that NeuN location in neuronal NM preparations matches almost perfectly the distribution of SRm160 in the same preparations (Fig. 3P). Therefore NeuN/Fox-3 is a reliable marker for speckles in neuronal cells. Moreover, the fact that a regulator of splicing such as NeuN/Fox-3 is concentrated in speckles bound to the ultimate neuronal NM is consistent with the fact that the brain is the organ where alternative splicing occurs more frequently [30].

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