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Characterization of NCAM diversity in cultured neurons

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A single transcript of the NCAM gene undergoes differential processing resulting in a multiplicity of mRNAs and their translation products. In this study, the diversity of NCAM in rat primary neuronal cultures was investigated utilizing immuno- and Northern blot analyses. NCAM polypeptides of 190 kDa (NCAM-A) and 135 kDa (NCAM-B) were shown to be associated with the neuronal phenotype. These data were confirmed by Northern blotting, which in both neocortical neurons and cerebellar granule neurons revealed mRNA classes of 7.4 kb and 6.7 kb encoding for NCAM-A and -B, respectively. However, oligonucleotide probes, specific for selected exons or exon combinations, revealed special features of cerebellar granule neurons as compared to neocortical neurons' expression of 4.3 kb NCAM mRNA, a relatively low amount of VASE-containing variants, and an apparent lack of mRNA species containing exons *a* and an AAG insert between exons 12 and 13. Distinct patterns of NCAM mRNA may putatively be related to the regional origin and functional specificity of the investigated neurons.

Neural cell adhesion molecule; Gene expression; Neuron; Cell culture; mRNA; Rat

1. INTRODUCTION

Induction and development of the neuronal phenotype is believed to be controlled by the concerted action of sets of morphoregulatory molecules, including the neural cell adhesion molecule (NCAM) [1,2].

The early expressed single NCAM gene exhibits a complex transcriptional pattern. Differential splicing of a 24 exon-containing pre-mRNA and alternative polyadenylation generate in rodents at least five size classes of mRNA (7.4, 6.7, 5.2, 4.3 and 2.9 kb) coding for NCAM [3,4]. Each of these possesses a complex coding potential [5–7], and the full range of possible diversity of the corresponding polypeptides has not yet been determined. In brain, the expressed polypeptides are grouped in three size classes of 190, 135 and 120 kDa, respectively referred to as NCAM-A, NCAM-B and NCAM-C. They mainly differ by their mode of attachment to membranes and the length of the cytoplasmic domain: NCAM-A possesses the longer cytoplasmic 'tail' as compared to NCAM-B, whereas NCAM-C lacks transmembrane and cytoplasmic domains and is linked to the membrane via a glycosylphosphatidylinositol (GPI) anchor [8,9].

The distribution of NCAM in the central nervous system is being extensively investigated. Immunocytochemical as well as in situ hybridization studies have indicated that the regional and cell type-specific expression of NCAM isoforms is developmentally regulated [5,10–14]. However, experiments utilizing a highly heterogeneous tissue such as whole brain or dissected parts, do not provide sufficient information on the capacity of specialized cell types of different regional origin for NCAM transcription and translation. On the other hand, the NCAM distribution in commonly used pure neuronal cell lines might significantly deviate from the original patterns [15]. Primary neuronal cultures may constitute more suitable models for the characterization of cell type-related expression of NCAM. Surprisingly, studies on neuronal cultures so far reported have not provided definite answers regarding the NCAM polypeptide pattern [8,10,11,16–18]. Moreover, a detailed analysis of the diversity of NCAM transcripts in neurons has not yet been undertaken.

Therefore, the present study was aimed at a characterization of the expression of different forms of NCAM in cultured neurons utilizing oligonucleotide probes specific for selected exons or their combinations. Data are presented on certain special features of NCAM mRNA expression in cultures of cerebellar granule neurons as compared to neocortical neurons.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

Monolayer cultures of fetal neocortical neurons were prepared from embryonic day 16 rat brain as described by Lyles et al. [19]. Granule neurons were cultured from cerebella of 7-day-old rats essentially as described by Schousboe et al. [20] and Drejer and Schousboe [21]. The

cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C and harvested for immuno- and Northern blot analyses after 4–7 days in vitro

2.2 *Antibodies and immunoblotting*

Polyclonal rabbit anti-rat brain NCAM (anti-NCAM) antibodies were prepared as described by Rasmussen et al. [22]. For immunoblot analysis, anti-NCAM antibodies purified by retroblotting [23] were kindly provided by Dr. Marianne Olsen (Protein Laboratory, University of Copenhagen) Samples solubilized as described previously [8] were boiled in SDS-PAGE sample buffer containing 5% β-mercaptoethanol and electrophoresed on a 7.5% polyacrylamide gel. Separated proteins were electrophoretically transferred from the gels onto Immobilon filters (Millipore, Bedford, USA) using a semi-dry blotting apparatus (Kem-En-Tec, Denmark). The blots were incubated with retroblotted anti-NCAM antibodies and the antigen–antibody complexes were visualized using alkaline phosphatase-coupled secondary antibodies (Dakopatts, Denmark).

2.3. *Oligonucleotides and Northern blot analysis*

Poly(A)$^+$ RNA was prepared as described previously [6], and probed by the radiolabelled synthetic oligonucleotides complementary either to selected sequences within specified exons (exons 7, 15, VASE), or to adjacent parts of the neighbouring exons (exons 7/8, 12/13, 12/a/AAG/13) These probes were designed using the published sequences of NCAM cDNA clones [3,24] and have previously been described by Andersson et al. [6]. Optimal conditions for the probe hybridization and washing were estimated using the computer program OLIGO (National Biosciences, USA) and adjusted experimentally Northern blot analysis was performed essentially as described previously [6].

3. RESULTS

In order to clarify the NCAM polypeptide pattern and its correlation with the observed sizes of the NCAM mRNA classes, immunoblotting of Triton X-100 extracts of cerebellar granule neurons and neocortical neurons was performed. Two discrete bands, corresponding to NCAM-A (190 kDa) and NCAM-B (135 kDa), were detected in neurons by means of retroblotted polyclonal antibodies raised against purified rat NCAM (Fig. 1, lanes 2 and 3). Expression of diverse splice variants of NCAM mRNA in neuronal cultures was studied by Northern blot analysis utilizing a series of radiolabelled synthetic oligonucleotide probes. The E7 probe specifically recognizes a sequence within exon 7, which is believed to be expressed in all NCAM mRNA species. In neocortical neurons, this probe hybridized to mRNA bands of 7.4, 6.7, 5.2 kb and, occasionally, to 2.9 kb. However, the signal at the 5.2 kb position was much weaker than the other bands. In cerebellar granule neurons the probe revealed mRNA size classes of 7.4, 6.7 and 4.3 kb. In contrast to the neocortical cultures, no signal was detected at the 5.2 kb position. There was no hybridization to mRNA from liver used as a negative control (Fig. 2, lane 2).

The E7/8 probe which is complementary to the adjacent sequences of exons 7 and 8, recognized two major bands of 7.4 and 6.7 kb in each of the analyzed neuronal cultures, and additionally a 4.3 kb mRNA in cerebellar granule neurons (Fig. 3A). Note, that using this probe

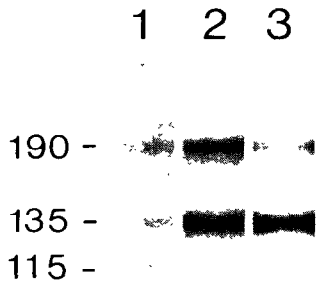

Fig. 1. Immunoblot analysis of NCAM polypeptides expressed in adult rat brain (lane 1), cerebellar granule neurons (lane 2), and neocortical neurons (lane 3). The relative molecular weights of the visualized bands are indicated in kDa at the left side

6.7 kb transcripts were labelled to a markedly higher extent than the 7.4 kb mRNA species.

The EVASE probe was utilized to identify the mRNA species containing the 30 base insert (exon VASE) between exons 7 and 8. Two bands (7.4 and 6.7 kb) of different intensities were observed in neocortical neurons (Fig. 3B). In contrast, cerebellar granule neurons under the present culture conditions expressed very low amounts of the VASE-containing transcripts: only prolonged exposure of the film allowed visualization of a weak signal associated with the 6.7 kb position (Fig. 3B).

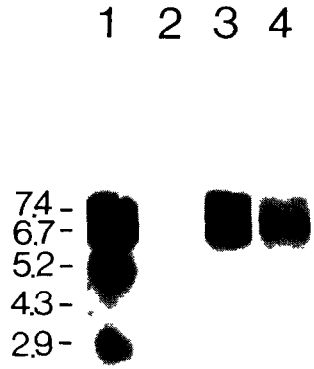

Fig. 2 Northern blot analysis of NCAM mRNA expression utilizing the E7 probe. Lane 1, postnatal day 40 rat brain (positive control); lane 2, liver (negative control); lane 3, neocortical neurons, lane 4, cerebellar granule neurons 10 μg of poly(A$^+$) RNA was loaded per lane The sizes of detected mRNAs are indicated in kilobases (kb) at the left side

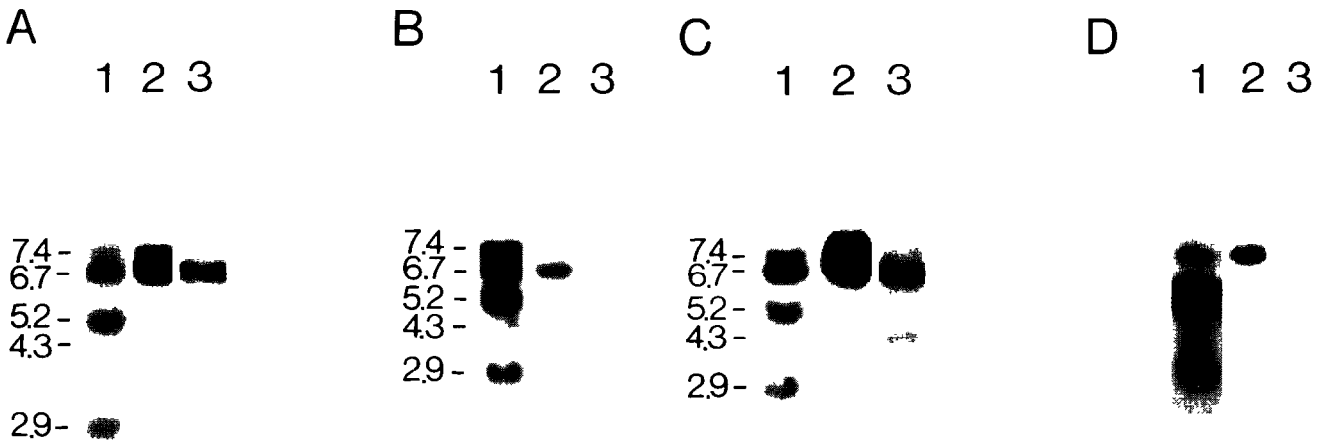

Fig. 3. Northern blot analysis of NCAM mRNA expression in rat brain (lane 1), neocortical neurons (lane 2) and cerebellar granule neurons (lane 3), utilizing oligonucleotide probes E7/8 (panel A), EVASE (panel B), E12/13 (panel C) and E12/*a*/AAG/13 (panel D). 10 μg of poly(A$^+$) RNA was loaded per lane. The sizes of detected mRNAs are indicated in kb at the left side of each panel.

The E12/13 probe, which covers the neighbouring regions of exons 12 and 13, hybridized to the 7.4 and 6.7 kb transcripts. Furthermore, cerebellar granule neurons apparently expressed also 4.3 kb mRNA species harbouring this exon combination (Fig. 3C).

The E12/*a*/AAG/13 probe is specific for mRNAs containing the block of the two adjacent small exons (exon *a* of 15 bases + AAG) inserted between exons 12 and 13. In cerebral neurons, this probe was found to bind to the 6.7 kb and, to a lesser extent, to the 5.2 and 2.9 kb mRNAs. No hybridization, even after long-term exposure, could be seen in cerebellar granule neurons (Fig. 3D).

4. DISCUSSION

A dynamic variability of the NCAM pattern is believed to be responsible for the fine tuning of the distinct developmental behaviour of specialized neural cells [2]. Thus, an accurate analysis of the cell type-specific spectra of the NCAM isoforms is one of the important prerequisites for the understanding of how the structural multiplicity may correlate with the diverse morphoregulatory activities of this molecule. The present study has examined the pattern of NCAM expression in different types of rat primary neuronal cultures.

Cerebellar granule neurons, as revealed by immunoblot analysis, synthesized polypeptides of two readily detectable size classes: 190 kDa and 135 kDa (Fig. 1). Previously, the doublets of NCAM-A and -B, but not NCAM-C, have been specifically attributed to neuronal phenotypes [8,16,25]. Recently, 140 kDa and, unexpectedly, 120 kDa polypeptides have been reported to be the major NCAM isoforms in cerebellar granule neurons [18]. In our experiments, a possible shedding [26] of the putatively pre-existing minor pool of NCAM-C under the present culture conditions could not a priori be ruled out. In order to clarify this controversial issue, the NCAM-encoding potential of the utilized cultured neurons was tested at the mRNA level. Furthermore, Northern blotting allowed an analysis of the presumed structural heterogeneity of neuronal NCAM, concealed within the readily detectable polypeptide bands.

In cerebellar granule neurons, none of the utilized probes revealed the 5.2 and 2.9 kb size classes of mRNA coding for NCAM-C [4], thus confirming the immunoblot pattern (Figs. 2 and 3). It should be noted that the 4.3 kb mRNA was only detected in cerebellar neurons, although the mode of generation of this transcript is unclear. Moreover, it is not known whether this message is translated. Recently, it has been shown that the 4.3 kb mRNA class contains sequences encoding for transmembrane forms of NCAM [6]. In fact, no band was observed by means of the probe recognizing exon 15, thought to encode the GPI-linked NCAM form (not shown). Interestingly, in neocortical cultures, the faint signal at the 5.2 kb position, as well as the occasionally detected 2.9 kb band, might be entirely attributed to mRNA populations containing the 18-base insert (*a* + AAG) between exons 12 and 13 (Figs. 2 and 3D). Neither the cellular origin, nor the coding potential of these transcripts could be clearly evaluated in the less homogeneous neocortical cultures. However, Lyles et al. [19] indicated that a faint, diffuse band at the region corresponding to NCAM-C could be detected in cultured neocortical neurons and suggested that this reflects a glial contamination. An intriguing difference

between the neuronal cultures employed in the present study was revealed by the probe E12/*a*/AAG/13: In cerebellar granule neurons, within the sensitivity range of the Northern blot analysis, it was not possible to detect mRNA species hybridizing to this probe (Fig. 3D). The translation product of the block of the alternatively spliced exons *a* and AAG is inserted between the two fibronectin type III-like domains of NCAM and might significantly alter the regional conformation [24]. Recently, protein fragments containing these domains have been shown to stimulate neurite outgrowth in cerebellar explants [27]. Pronounced neurite outgrowth-promoting and signal-transducing abilities are also attributed to NCAM-B isoforms lacking the product of VASE [28]. Our data suggest that in cerebellar granule neurons the VASE-containing transcripts are expressed to a lesser extent than in neocortical neurons. The revealed pattern hints at the putatively higher potential of the employed cerebellar granule neurons for differentiation in vitro. Taking into account the developmental stage of the cerebellar neurons at the start of culturing [29] and the age of the cultures analyzed, the above suggestion seems to be partially consistent with recent data indicating the downregulation of NCAM in migrating postmitotic granule neurons [30]. It is noteworthy that cerebellar granule neurons utilize glutamate as a neurotransmitter, whereas neocortical cultures, although being functionally less homogeneous, are enriched with GABAergic interneurons [31]. It is possible that expression of the NCAM isoforms is differentially modulated by the distinct neurotransmitters and other putatively secreted factors affecting intracellular second messenger systems. For example, transmitter-mediated downregulation of the NCAM-related cell adhesion molecule (ApCAM) has been demonstrated in *Aplysia* sensory neurons [32]. Furthermore, a cAMP-dependent regulation of NCAM expression in astrocytes has recently been demonstrated [33]. Further experiments utilizing transfection techniques and functional tests are required to clarify the significance of an in vitro pattern of NCAM expression as well as the roles of individual NCAM isoforms in the development of neuronal networks.

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