Short communication

Neurogranin is locally concentrated in rat cortical and hippocampal neurons

Martin Neuner-Jehle a, Jean-Pierre Denizot b, Jacques Mallet a, *

a Laboratoire de Génétique Moléculaire de la Neurotransmision et des Processus Neurodégénératifs, C.N.R.S., Bât. CERVI, Hôpital de la Pitié Salpêtrière, 83, bd de l'Hôpital, F-75613 Paris, France
b Institut A. Fessard, C.N.R.S., 91198 Gif-sur-Yvette Cedex, France

Accepted 25 June 1996

Abstract

The rat protein kinase C substrate neurogranin has a granular distribution in cortical and hippocampal neurons. We demonstrate that in these cells, granular labelling corresponds to a local concentration of neurogranin-immunoreactivity at both the membranes of mitochondria and trans-Golgi vesicles and soma-proximal dendritic shaft and spinal head structures. Our findings suggest that the function of neurogranin could be affected by protein assembly at these cellular sites.

Keywords: Calmodulin; Dendritic spine; Golgi complex; Mitochondria; PKC; RC3

Neurogranin (Mr = 17 kDa; NG, RC3, BICKS) [7,20,28,29] is a neuron-specific rat protein, homologous to the bovine protein p17 [2]. It has recently been recognized as an in vivo substrate for both the α-, β-, and γ-isomers of the serine/threonine protein kinase C (PKC) [4,12] and the synapse-associated Ca2+-dependent phosphorylase kinase [18]. These kinases phosphorylate the Ser-36 residue of NG in the IQ domain [5] which comprises a calmodulin (CaM) binding motif [3,18,27]. As shown in vitro, an addition of a negatively charged phosphate to Ser-36 reduces the affinity of NG to Ca2+-free CaM [10]. This adds to the notion that NG is a cellular CaM regulator, linking protein kinase-dependent processes with CaM-mediated pathways [11,16]. Several potential cellular sites for the action of NG have been identified in rat striatal neurons [29]. NG is dispersed in cell bodies but associated with cell nuclei, dendritic spines, and some myelinated axons. Thus, in the striatum, NG may act in a variety of cellular compartments. In cortical and hippocampal cells, NG-immunolabelling has been reported to be often granular and associated with yet unspecified cellular structures [20,28]. To identify these structures, we analyzed the subcellular distribution pattern of the protein in NG-enriched rat brain areas including layer II of the parietal cerebral cortex and the hippocampal area CA1 [17,20].

Three male Sprague-Dawley rats (200–300 g b.wt; kept under a 12 h light/12 h dark cycle) were anaesthetized (0.4 g chloral hydrate/kg b.wt; i.p.) and transcardially perfused (fixative: 4% (w/v) paraformaldehyde, 15% (v/v) picric acid, 0.15 M phosphate buffer pH 7.4 [25]) at light-onset. 60 μm frontal sections were cut from their brains with a vibratome and postfixed overnight at 4°C in the same fixative containing 1% (v/v) glutaraldehyde. Freely floating sections were then exposed to 0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS) for 1 h at ambient temperature and incubated overnight at 4°C with affinity-purified anti-NG antibodies (10 μg/ml PBS, 2% (v/v) normal goat serum) [17]. Immunoreaction was carried out with the Vectastain Elite Kit (Vector Laboratories), 3,3′-diaminobenzidine (DAB, Sigma), and hydrogen peroxide according to the manufacturer’s instructions. Control sections of the same brain regions were similarly treated but not incubated with anti-NG antibodies. After overnight fixation at 4°C with 0.25% (w/v) osmium tetroxide and 0.75% (w/v) potassium-perchlorate in 0.1 M phosphate buffer (pH 7.8), DAB-stained and control sections were embedded in Spurr’s resin (TAAB). 800 Å slices were cut from their immunoreactive surface using an ultratome (LKB), mounted on copper grids, and inspected under an electron microscope (Philips CM10). Where indicated, ultrathin slices were counterstained at ambient temperature with saturated solutions of uranyl acetate (Pro-labo) for 30 min and lead citrate (ICN Pharmaceutical) for 3 min [21].
The specificity of immunostaining for NG protein was verified by light microscopy on sections of three rat brains. The distribution of NG-immunoreactivity (IR) is similar in all rats examined. In particular, layer II neurons of the parietal cerebral cortex and pyramidal cells of the hippocampal area CA1 revealed intense NG-IR in both neu-
ronal perikarya and the soma-proximal region of their dendrites (Fig. 1A and Fig. 2A). This pattern is identical to the previously described distribution of both NG mRNA and NG protein [17,20,22,28,29], indicating that the affinity-purified anti-NG antibodies used specifically recognize NG protein in rat brain sections.

At the subcellular level, the NG-IR distribution patterns in both cortical layer II neurons and hippocampal CA1 pyramidal cells are similar in all rat brains examined. In these neurons, dispersed electron-dense NG-deposits are confined to peripheral cytoplasmic sites of cell somata whereas the perikaryal plasma membrane is devoid of immunolabelling (Fig. 1C and Fig. 2C). In close association with regions of dispersed staining, which may correspond to cytoplasmic sites of a non-aggregated NG protein form, a local concentration of electron-dense immunoreaction deposits was observed near and at the membrane of particular hippocampal mitochondria (Fig. 2C, arrow heads). Membranous labelling of mitochondria is apparent also in cortical cells (Fig. 1C, double arrow) but not in control sections of either brain region (Fig. 1B and Fig. 2D). Furthermore, membranous immunolabelling was frequently detected at both the Golgi apparatus (GA) and the endoplasmic reticulum (ER) of cortical and hippocampal neurons. At the GA, electron-dense immunoreaction is confined to the trans-Golgi system where it is associated mainly with membranes of vesicular structures (Fig. 1C,D and Fig. 2C; arrows). This observation is consistent with a previous report of the presence of NG in microsomal rat brain preparations [30]. It also indicates that the protein may serve as a substrate for the Golgi-associated α- and β-isoforms of PKC thought to be involved in vesicle trafficking [13,23]. At the ER, membranous labelling is less pronounced and was observed also in control sections, indicating that the ER membrane may be non-specifically stained. Electron-dense immunolabelling has been found neither within mitochondria nor in the lumen of both the GA and the ER. Similarly, the cell nuclei of cortical and hippocampal neurons and their nuclear membranes are devoid of NG-IR. The local concentration of NG in particular perikaryal compartments in both brain regions contrasts with the previously reported dispersed NG-immunolabelling in neuronal somata of the rat striatum [29]. It further suggests that cellular protein accumulation is characteristic of cortical and hippocampal neurons. As it has been proposed for NG, a glycine-rich sequence at the carboxy-terminus may mediate multimeric protein assembly [28]. Moreover, NG protein assembly could also depend on ADP-ribosylation which inhibits polymerization of other proteins such as tubulin [24] and non-muscle actin [1]. Indeed, NG has recently been found to be ADP-ribosylated near its PKC phosphorylation site [7]. Such reversible post-translational protein modification may account for the observed co-localization of the aggregated and dissociated forms of NG in cortical and hippocampal neurons. By introducing a net negative charge into the CaM binding region of NG, ADP-ribosylation may also influence the protein’s binding affinity to Ca2+-free CaM. Possibly, ADP-ribosylation-dependent protein assembly regulates the availability of NG for protein kinase- and CaM-mediated cellular reaction pathways.

Similar to the pattern of a locally confined perikaryal immunolabelling, electron-dense NG-IR is also concentrated within both dendritic shaft and spine compartments. In dendritic shafts of hippocampal pyramidal cells, NG-IR is apparent at and close to filamentosous structures of the cytoskeleton (Fig. 2B; arrows). It is unclear whether NG expresses its function at these sites or is transported to other cellular compartments. Highest levels of dendritic NG-IR were observed in particular spine heads of cortical layer II (Fig. 1E) and apical hippocampal CA1 dendrites (Fig. 2E). The spinal immunoreactivity is consistent with a previous report of the presence of NG in synaptosomal rat brain preparations, as determined by immunoblotting [30]. Spinal NG-IR is associated mainly with soma-proximal axospinal symmetric synapses whereas axosomal junctions and shaft synapses are devoid of immunolabelling. In immuno-positive spines, NG-immunolabelling is particularly strong within the cytoplasm of their head compartments, in postsynaptic densities, and on the subsynaptic membrane (Fig. 1C,E and Fig. 2E). This post- and subsynaptic staining pattern resembles that of NG-positive dendritic spines of asymmetric synapses in rat striatum [29]. The presence of NG in both cytoplasmic and membranous cellular environments is consistent with both the detection of the protein in soluble and membranous protein fractions of the rat cerebral cortex and an association of NG with insoluble synaptosomal protein fractions [3,11,30]. In the cytoplasm of immuno-positive spine heads, NG-IR is often granular. As described for neuronal perikarya, an accumu-

**Fig. 1.** NG-immunostaining in cell bodies and soma-proximal dendritic synapses of cortical layer II neurons. A: under a light-microscope, NG-IR is visible mainly in cell somata and their soma-proximal dendritic regions (arrows). B: in a control section, neither the cell nucleus (nu) nor mitochondria (mi) are immunolabelled. The membrane of the endoplasmic reticulum (er) appears to be more electron-dense than other membranous structures. C: near the perikaryal plasma membrane (asterisk), immunolabelling is closely associated with membranes of both mitochondria (mi; double arrow) and vesicles of the trans-Golgi apparatus (ga; arrow) whereas the cell nucleus (nu) and the endoplasmic reticulum (er) are devoid of immunoreactivity. Near the NG-positive cell body, dendritic spine heads are depicted showing intense granular immunostaining (arrow heads). D: close to the perikaryal plasma membrane, granular NG-immunolabelling is associated with a Golgi-like tubular structure (arrow). A myelinated axon (ax) nearby is devoid of NG-IR. E: on a counterstained ultrathin slice, granular NG-IR is detected in a dendrite spine head, notably in the cytoplasm, the postsynaptic density (arrow head), and on the subsynaptic membrane (double arrow). Together with an unlabelled dendritic spine nearby (asterisk) both spines form synapses with an unlabelled axonal bouton (ab). Brain sections: 60 μm (A), 800 Å (B–E). Scale bars: 50 μm (A), 600 nm (B–D), 180 nm (E).
lution of NG-IR in dendritic spines could be caused by protein assembly which may affect the availability of NG for PKC-dependent and CaM-mediated second messenger pathways [8,10]. Alternatively, granular immunoreaction could represent sites of mRNA translation at dendritic or spinal polyribosomes [6,26]. Evidence for dendritic mRNA expression has previously been provided by in situ hybridization histochemistry in rats showing NG mRNA in soma-proximal dendrites of both hippocampal pyramidal and granule cells and cortical neurons [15]. Moreover, both variants of NG mRNA (1.0 kb, 1.4 kb [22,28]) were detected in mossy-fiber synaptosomes of the rat hippocampal area CA1 [6], suggesting that both variants can be expressed in spinal compartments. Unlike dendritic spines, presynaptic terminals and myelinated axons of cortical and hippocampal neurons are devoid of electron-dense NG-immunoreaction deposits. This is in contrast to the rat striatum where NG-IR has been detected in both presynaptic boutons of striatal neurons and myelinated corticofugal axons [29].

The specificity of NG-IR for a subpopulation of soma-proximal spine heads indicates that NG acts at synapses with particular functional properties. Long-term potentiation (LTP) of synaptic activity, for instance, leads to an increase of NG phosphorylation in the CA1 region of rat hippocampal slices [14,19]. Moreover, an injection of anti-NG antibodies into cultured rat hippocampal CA1 pyramidal cells prevents the establishment of tetanus-evoked LTP [9]. The protein may therefore play a critical role in regulating postsynaptic processes of LTP. With respect to the observed specific spinal distribution pattern of NG in the rat cerebral cortex and hippocampus, it is possible that the protein accumulates in dendritic spines which are subjected to LTP-related changes in synaptic activity. This notion further implies that NG could serve as a neuronal marker for synaptic plasticity processes.

Acknowledgements

We thank Jean-Paul Bouillot and Denise Champion for photographic assistance. The work was supported by a grant from the Swiss National Research Foundation to MNJ.

References


