Neurogranin: Immunocytochemical Localization of a Brain-Specific Protein Kinase C Substrate

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The developmental expression and the cellular localization of neurogranin (formerly designated p17), a brain-specific protein kinase C (PKC) substrate, were investigated. The developmental expression of neurogranin was studied by immunoblotting of rat brain and neuronal cell-culture extracts using neurogranin polyclonal antibodies. Neurogranin synthesis was found to be developmentally regulated, with no expression in the embryonic and neonatal period and an abrupt increase between 2 and 3 weeks of age. By immunohistochemistry, neurogranin was found essentially in the adult rat telencephalon, specifically located in the cell bodies and dendritic processes of neurons of the cerebral cortex, hippocampus, striatum, and a few other discreet areas. Neurogranin immunoreactivity was nearly absent in the thalamus, cerebellum, and brain stem. The late developmental expression and the dendritic localization of neurogranin in neurons are 2 features that also characterize the type I PKC isozyme. The specific localization of the protein in integrative areas of the rat brain suggests a highly specialized function of neurogranin in the CNS. A possible role for neurogranin in the transduction of the PKC activation signal at the postsynaptic level is suggested.

Protein kinase C (PKC), a Ca²⁺-activated phospholipid-dependent kinase, is the receptor for tumor-promoting phorbol esters and is thought to play an important role in controlling several cellular processes (see Nishizuka, 1988, for review). PKC is present ubiquitously in a variety of tissues and is especially concentrated in the brain (Huang et al., 1986). Three different PKC isozymes, designated types I, II, and III, have been described (Huang et al., 1986). They are products of γ-, β-, and α-type genes, respectively (Cousens et al., 1986; Brandt et al., 1987). In situ hybridization and direct immunohistochemical studies revealed differences in the distribution and developmental expression of the 3 PKC isozymes in the brain, suggesting that the different isozymes of PKC have distinct functions (Brandt et al., 1987; Huang et al., 1987; Hashimoto et al., 1988; Yoshida et al., 1988).

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Materials and Methods

Purification of proteins. Bovine brain neurogranin (p17) was purified as previously described (Baudier et al., 1989). For rabbit immunization, a final purification step was included, consisting of reverse-phase chromatography on a Pro RPC column. Neurogranin was eluted at 0.5 ml/
min with a 50-m1 linear gradient of 0-50% acetonitril with 0.1% tri-
fluoroacetic acid. Neurogranin eluted at about 20% acetonitril. The
protein was then extensively dialyzed against 20 mm Tris-HCl (pH, 7.5)
plus 10 mm dithiothreitol (DTT) and 1 mm EDTA and stored at -20°C.
Bovine brain neuromodulin and r proteins were prepared as previously
described (Baudier et al., 1987, 1989).

Production of antibodies. Antibodies against purified neurogranin,
neuromodulin, and r proteins were prepared in New Zealand white
rabbits. The proteins (300-350 kg) were emulsified in complete Freund's
adjuvant and injected intradermally at multiple dorsal sites. At 2-3-
week intervals, the rabbits were given 2 other booster injections of 150-
250 kg proteins in incomplete Freund's adjuvant. The rabbits were bled
repeatedly, and the antibody titers were determined by Western blotting.
Antisera that showed significant activity at 1:1000 dilution were col-
lected. A second type of rabbit antisera against neurogranin was ob-
tained by injecting the neurogranin monomer, which was cut directly
from the SDS-polyacrylamide (0.1% 12.5%) gels and emulsified in in-
complete Freund's adjuvant. This antisera gave similar immuno-
staining patterns on rat-brain sections to the first antisera (see Results).

Purified antibodies against neurogranin were obtained by affinity chro-
matography of purified serum IgG on neurogranin coupled to a glu-
arable-activated Triscylcolum!.

Tissue and cell extracts. Tissues were freshly excised from rats killed
deprivation. The tissues (0.5 gm/ml) were homogenized in buffer
A [100 mm Tris-HCl (pH, 7.4), 2 mm EDTA, 2 mm DTT, 100 mm
NaCl, 1% Triton X-100] using a Dounce homogenizer, and the extract was clarified by centrifugation. Perchloric acid was added to the
supernatant (final concentration 2.5%), which was immediately centri-
figated for 15 min at 15000 x g. The supernatant was then dialyzed
overnight against 40 mm Tris-HCl (pH, 6.9), 50 mm NaCl, and 2 mm
DTT. Cortical neuronal cell cultures were obtained from fetal rats, as
previously described (Gensburger et al., 1986). The cultures were rinsed
3 times with PBS. The cells were disrupted by brief sonication in buffer
A and processed in the same way as the tissue extract.

Immuno blotting. Tissue and cell extracts were electrophoresed in 0.1%:
12.5% SDS-polyacrylamide gels, then transferred electroblotically to
an Immobilon blotting membrane (Millipore). After transfer, the mem-
brane was incubated 30 min with 3% gelatin in Tris-buffered saline
(TBS) buffer [20 mm Tris-HCl (pH, 7.5) and 0.5 mm NaCl]. After being
washed with TBS, the blotting membrane was incubated overnight at
room temperature with primary antibodies diluted 1:1000 in TBS
buffer (TBS buffer + 0.05% Tween 20) containing 1% gelatin, followed by
incubation for 2 hr with alkaline phosphatase-conjugated goat anti-
rabbit antibodies diluted with TBS and 1% gelatin buffer. Inmunore-
active bands were detected using 5-bromo-4-chloro-3-indolyl phos-
phate (0.15 mg/ml) and Nitro blue tetrazolium (0.3 mg/ml) in 40 mM
phosphate buffer (pH, 9.8) and 3 mm MgCl2.

Immunohistochemistry. Male Sprague-Dawley rats (2-3 months old, n =
6) were anesthetized by intraperitoneal injection of pentobarbital and
perfused through the left ventricle with 50 ml 0.9% NaCl, followed by
100 or 300 ml of 1 of the 4 fixative solutions: (1) 4% paraformaldehyde
used for immunohistochemical experiments. All steps were performed
with gentle agitation of the sections. The sections were incubated in primary neurogranin antisera at dilutions 1:100, 1:200, 1:500, or 1:1000
in phosphate/NaCl buffer plus 10% normal sheep serum for either 2 or
12 hr at room temperature. The sections were washed for 1 hr in 6-9
changes of phosphate/NaCl buffer, then incubated 2-3 hr with peroxi-
diside-conjugated goat anti-rabbit IgG (diluted 1:200 in phosphate/NaCl
buffer plus 10% normal sheep serum). The last wash step employed 6-9
changes of phosphate/NaCl buffer. The peroxidase activity was de-
veloped for 10-15 min with 0.018% 4-chloro-1-naphthol plus 0.03% hydrogen peroxide in phosphate/NaCl buffer. Immunocytochemical controls were performed using normal rabbit serum or immunosera
on preabsorbed with the purified protein (0.1 mg/ml).

To study the general distribution of neurogranin, we used different
fixatives and antibodies to minimize the possibility of selective regional
reduction of the antigenicity (see below), and 2 different rabbit antisera
were used. In all cases, the staining pattern for neurogranin was highly
reproducible. The use of affinity-purified IgG against neurogranin gave
a similar staining pattern to that of the crude antisera.

Results

Tissue distribution and developmental expression of
neurogranin

The specificity of the polyclonal antibodies raised against pure
neurogranin can be seen from transblot analysis of total and
purified rat-brain extracts (Figs. 1, 2). The major band
detected in the total adult-rat-brain extracts migrated with the
same apparent molecular weight as that of the purified bovine
protein, indicating that neurogranin is not a proteolytic fragment
of a larger molecule generated during purification. Although a
sequence homology has been found between neurogranin and
neuromodulin, polyclonal neurogranin antibodies did not cross-
react with neuromodulin and vice versa (data not shown).

The tissue distribution of neurogranin in adult rat tissues was
studied by immunoblotting on homogenates treated with 2.5%
perchloric acid as described in Materials and Methods. Neu-
rogranin was found only in the cerebrum, not in the cerebellum
or other tissues (Fig. 1).

The changes in the levels of neurogranin during brain post-
natal development were analyzed by immunoblotting of crude
rat-brain extracts (Fig. 2A). In the newborn rat brain, no neu-
rogranin immunoreactivity could be detected after immuno-
blotting of brain extracts, suggesting that the synthesis of the
protein occurs only after birth. The postnatal expression of neu-
rogranin appeared to be very low up to 1 week of age, then a
rapid increase was observed between 2 and 3 weeks. In addition,
we did not find immunohistochemical labeling of brain struc-
tures in rats prior to the second postnatal week (results not shown).

We also investigated the expression of neurogranin in primary
cell cultures derived from cerebral hemispheres of rats at
different embryonic ages and harvested at different periods of time
(Fig. 2B). The results were compared with those for neuromod-
ulin and the microtubule-associated r protein (Fig. 2C,D).
In contrast to neuromodulin and r protein, neurogranin was only
present in advanced differentiated neurons (adult rats), but not in
less mature cultured neurons.

To compare the abundance of neurogranin with that of neu-
immunocytochemical localization of neurogranin

A

Figure 2. Developmental expression of neurogranin in rat brain tissue. A, Total brain extracts of newborn rats (0) and from rats at different postnatal ages, as indicated, were analyzed by immunoblotting. The immunoreactive bands correspond to the neurogranin monomer. In each lane, the proteins loaded correspond to 10 μg tissue. B-D, Comparison of the expression of neurogranin (B), neuromodulin (C), and τ proteins (D) in primary cortical neuronal cell cultures derived from brains of rats at embryonic day 14 (E14) harvested after 7 d (lane 1) and E17 harvested after 4 d (lane 2). Percoll-acid-treated cell extracts were analyzed by immunoblotting using antibodies (1:1000 dilution) against neurogranin (B), neuromodulin (C), and τ proteins (D). Ad, adult rat brain extract. E, Comparison of the neurogranin and neuromodulin antigens in Triton X-100-solubilized extracts of rat cortex (Cx), cerebellum (Cb), hippocampus (Hip), and thalamus (Tha) by Western blots. In each lane, the proteins loaded correspond approximately to 2.5 μg tissue. The proteins were first separated by SDS-polyacrylamide (0.1%; 12.5%) gel electrophoresis, then transferred electrophoretically to an Immobilon blotting membrane, and the membrane was incubated with a mixture of neurogranin and neuromodulin antibodies (1:200 dilution) for 2 h. Arrowheads indicate position of colored molecular-weight markers (m.w.): 14.3 kDa, 21.5 kDa, 30 kDa, 46 kDa, and 68 kDa. Arrows indicate position of neuromodulin (N) and neurogranin (n).

romodulin in different regions of the adult rat brain, we analyzed the presence of these 2 proteins on the same Western blot of Triton X-100-solubilized extracts of the cortex, thalamus, hippocampus, and cerebellum (Fig. 2E). Neuromodulin immunoreactivity was found abundant in the 4 extracts, while neurogranin appeared as minor immunoreactive bands only in cortical and hippocampal extracts. These data indicate that neurogranin is, therefore, present at a much lower concentration than neuromodulin in the adult rat brain.

Regional distribution of neurogranin in the adult rat brain

Figure 3A shows typical immunocytochemical staining of neurogranin in a parasagittal section of an adult rat brain. Figure 3B schematically depicts the relative intensity of neurogranin immunoreactivity in some brain structures. The highest immunoreactivity was found in certain layers of the hippocampal formation and the neocortex. Medium staining densities were found in the striatum, colliculi, pyriform cortex, olfactory tu-
Figure 3. Neurograninlike immunoreactivity in parasagittal sections of adult rat brain (80 µm). A. The brain of a 2-month-old rat was fixed in 4% paraformaldehyde and 0.1% glutaraldehyde. Slices were incubated with a 1:100 dilution of rabbit polyclonal antibodies against neurogranin for 12 hr at room temperature, and the immunoreactivity was revealed by the peroxidase method as described in Materials and Methods. B. Semi-quantitative distribution of neurogranin; high, medium, and low immunostaining densities in various areas are indicated. See Appendix for abbreviations.

Figure 3. Neurograninlike immunoreactivity in parasagittal sections of adult rat brain (80 µm). A. The brain of a 2-month-old rat was fixed in 4% paraformaldehyde and 0.1% glutaraldehyde. Slices were incubated with a 1:100 dilution of rabbit polyclonal antibodies against neurogranin for 12 hr at room temperature, and the immunoreactivity was revealed by the peroxidase method as described in Materials and Methods. B. Semi-quantitative distribution of neurogranin; high, medium, and low immunostaining densities in various areas are indicated. See Appendix for abbreviations.

bercle, and some amygdala nuclei. Neurogranin immunoreactivity was almost completely absent in the thalamus, cerebellum, brain stem, and spinal cord. The dark appearance of the outer surface of the cerebellum and of the middle part of the corpus callosum sandwiched between the hippocampus and the overlying cortex seen in Figure 3A is not immunochemical staining. This is a photographic artifact due to the brightness of the flash light on the sides of the slice.
Figure 4. Distribution of neurogranin in coronal (7-μm) section of hippocampal formation. A and B, In the fascia dentata, neurogranin immunoreactivity is found almost exclusively in the granular cells; the soma appears well stained, whereas the dendrites appear poorly immunoreactive. The interneurons (located in the hilus) are rarely immunoreactive. C and D, Distribution of neurogranin in the CA3 field; pyramidal cells show high density of immunostaining in the soma and the apical dendrites (in the stratum lucidum), whereas the basal dendrites are devoid of immu-
Observations at higher magnification indicated that the staining was restricted to neurons in all neurogranin-positive regions. No staining of glial cells could be observed. The staining was predominantly found in the perikarya and the dendrites of pyramidal neurons; axons were not labeled. The nuclei of the neurons appear rarely immunoreactive, except in the cerebral cortex (see Fig. 5C).

The same immunocytochemical staining pattern was observed with serum dilutions from 1:100 up to 1:1000 and with affinity-purified antibodies. Only the intensity of the staining decreased with the higher serum dilutions. The specificity of the immunostaining was further confirmed by preadsorption of the anti-neurogranin serum with the purified protein. The immunostaining was totally inhibited when the serum was preincubated for 4 hr with purified neurogranin (0.1 mg/ml; data not shown but provided to referees for examination).

Figures 4–8 illustrate in detail the distribution of neurogranin in the various structures and the localization at the cellular level.

In the hippocampal formation (Fig. 4), most of the pyramidal neurons of Ammon’s horn and the subiculum and the granular cells of the fascia dentata were conspicuously stained: only a few putative interneurons in the hilus (Fig. 4A) and the stratum oriens of Ammon’s horn (Fig. 4C,E) showed a positive immunostaining. As shown in Figure 4B,D, and F, neurogranin immunostaining clearly depicted the perikarya of both pyramidal and granular cells, whereas only the apical dendrite of pyramidal cells showed dense immunostaining; the basal dendrites of pyramidal cells and the dendritic arborization of granular cells showed fair immunostaining. There were no signs of staining in axons; that is, in the lucidum layer, occupied by the mossy fibers, only dendrites appeared stained. The alveus, occupied by the axons of pyramidal cells, also appeared devoid of immunodeposits.

The cerebral neocortex (Figs. 3, 5, 6), with the hippocampal complex, showed the highest density of immunolabeling. The entire cortex was well stained, including the pyriform (Fig. 6B), entorhinal (Fig. 6D), cingular, temporal, frontoparietal (Fig. 5A,C), and striatal cortices (Fig. 5B,D), with a slight predominance in the striatal and frontoparietal cortices. The immunolabeling has a characteristic laminar pattern, with the highest neuronal immunostaining in layers II–IV and VIa (Fig. 5A,B,D). In these layers, the perikarya and the dendritic trees, particularly the long apical dendrites of the pyramidal cells, appeared intensely stained. Occasionally, the nucleus of pyramidal and nonpyramidal neurons were also stained (Fig. 5C). In layer V, only a few large pyramidal cells appeared slightly immunolabeled. The immunoreaction clearly stained the terminal arborization of pyramidal dendrites in layer I.

In the amygdala (Fig. 6), most of the different nuclei appeared stained by the neurogranin antibodies; only the medial nuclei was devoid of cellular staining. The strongest immunostaining was found in the basolateral, anterior cortical, and central amygdaloid nuclei; in contrast, the basomedial, intercalated, and amygdalohippocampal areas were moderately stained.

The basal ganglia (Figs. 3, 7) also appeared heterogeneously stained. The highest density of immunolabeling was found in the soma of neurons of the caudate putamen (Fig. 7). The claus-
Figure 5. Micrographs showing the distribution of neurogranin in rat neocortex (7-µm section). A, Coronal section of the frontal cortex (25×). B and D, Coronal section of the striatal cortex (62.5×). C, Horizontal section of the frontoparietal cortex (125×). Note the high immunostaining of the cellular layers II-IV; the soma and the apical and basal dendrites of pyramidal cells are densely stained; some neurons show a clear staining of their nucleus.
Figure 6. Diagrams (A, C) and coronal sections (B, D, E) showing distribution of neurogranin immunoreactivity at level of amygdala. The strongest labeling is found in the basolateral, cortical, and amygdalocortical nuclei. B and D also show the presence of immunostaining in the entorhinal and primitive olfactory cortices. B and D, 25×; E, 62.5×. See Appendix for abbreviations.
Figure 7. Neurogranin immunoreactivity in caudate putamen. Immunostaining is found exclusively in the soma of neurons; the dendrites and axons are devoid of immunodeposits. Arrows indicate a few nonimmunoreactive neurons. A, 25×; B, 62.5×; C, 125×.

brain, type II and type III PKC are already present at birth, increase rapidly, and reach a plateau level at 2 weeks. Furthermore, in adult-rat-brain sections, neurogranin has a similar immunostaining pattern to type I PKC (Nishizuka, 1988; Saito et al., 1988), whereas it differs from the distribution of type II and III PKC (Huang et al., 1988; Hosoda et al., 1989; Saito et al., 1989). Moreover, electron microscopic studies on the cellular localization of β₁ PKC (type II) suggested a preferential association of these subspecies with the Golgi complex (Saito et al., 1989). Finally, Brandt et al. (1987), by contrasting the locali-
zation of PKC proteins, PKC mRNA, and phorbol ester bindings, have suggested that PKC-I proteins are located postsynaptically (as is neurogranin), whereas PKC-II and PKC-III proteins are located presynaptically in the cerebellum and the striatognal fibers. Recently, Huang (1989) reported that, in the cerebellum, even though PKC-I and PKC-III colocalize in Purkinje cells, only PKC-I, not PKC-III, is present in the dendrites of these cells, suggesting a function for PKC-I in postsynaptic signal transduction (Huang, 1989). However, several studies suggested a preferential postsynaptic localization of all 3 PKC isoforms in the hippocampus and neocortex (Worley et al., 1986; Huang et al., 1988; Saito et al., 1988, 1989). Work is in progress to analyze in vitro the kinetics and other parameters of neurogranin phosphorylation by the different PKC isozymes, which may help to understand the substrate specificity of each isoform and the physiological functions of these enzymes.

It has been shown that activation of PKC can mimic the biophysical effects of associative learning on neurons, and that associative memory induces activation and translocation of the PKC from the cell soma to the dendrites of the CA1 pyramidal cells of the hippocampus (Olds et al., 1989), where endogenous substrates will support the transduction of the PKC activation signal. PKC activation is also necessary for the maintenance of long-term potentiation (LTP) (see Linden and Routtenberg, 1989, for review), a synaptic model of memory produced by a brief train of high-frequency electrical stimulation (Bliss and Lomo, 1973) or by phorbol esters (Malenka et al., 1986; Aniksztejn et al., 1987). Moreover, in the CA1 region of the hippocampus, intracellular delivery of PKC inhibitors blocks LTP induction but not its expression, suggesting that postsynaptic PKC is necessary for the induction of LTP (Malinow et al., 1989). To our knowledge, neurogranin is the first postsynaptic PKC substrate identified to date that is specific to soma and dendrites and is abundantly present in areas of the adult rat brain that are capable of long-lasting enhancement of synaptic transmission, notably, the hippocampus and neocortex (Iriki et al., 1989). Further studies on a possible role of neurogranin in the transduction of the PKC activation signal in synaptic plasticity have to be considered.

It is also significant that neuromodulin (also called F1, B50, and GAP43), which is found highly concentrated in neuropil layers (i.e., the stratum oriens and stratum radiatum of the CA1 area and the molecular layer of the fascia dentata), seems to be located presynaptically (Snipes et al., 1987; MacGuire et al., 1988). Activation of a presynaptic protein kinase and phosphorylation of neuromodulin could also be necessary for the maintenance of LTP (Linden and Routtenberg, 1989; Malinow et al., 1989); nevertheless, the type of PKC involved is presently unknown, because, at least in the CA1 area, the defined PKC isozymes are all postsynaptically located (Worley et al., 1986; Hvang et al., 1988; Saito et al., 1988, 1989).

Finally, in addition to its possible functional importance, the neurogranin antigen may also serve as a specific marker of neuronal cell types, particularly suitable in the hippocampus, where it is found almost exclusively in the pyramidal and granular cells, but not in interneurons.

Appendix

| Acb | accumbens nucleus |
| Aco | anterior cortical amygdaloid nucleus |
| AHi | amygdalohippocampal nucleus |
| Bl  | basolateral amygdaloid nucleus |
| Blv | basolateral amygdaloid nucleus, ventral |
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