Short Communication

Neurensin-1 expression in the mouse retina during postnatal development and in cultured retinal neurons

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Neurensin-1/Neuro-p24 (previously named Neuro-p24) is a neuron-specific membrane protein that is localized particularly in neurites. Neurensin-1 is considered to play an essential role in neurite extension during nervous development, regeneration and plasticity. To understand what role Neurensin-1 plays in retinal differentiation, we examined Neurensin-1 distribution and gene expression pattern in the postnatally developing retina of the mouse, because the retina is an excellent model for nervous development. In the postnatal day (PD) 1 retina, intense Neurensin-1 immunoreactivity was found in the optic nerve fiber layer. Faint staining was seen in the ganglion cells, presumptive amacrine and horizontal cells. As the postnatal development proceeded, the optic fibers became more intensely stained in addition to other parts of the retina such as the ganglion cells, inner plexiform layer and horizontal cells. As the retina developed further to attain maturity, the staining in the retina became less pronounced, although the optic nerves remained positively stained. The distribution of Neurensin-1 mRNA was consistent with these results and confirmed that the ganglion, amacrine and horizontal cells actively synthesize Neurensin-1 in the developing retina. In the retinal cell culture from newborn mice, two types of neural cells were stained for Neurensin-1, one of which showed long processes and appeared presumptive ganglion cells. These results suggest that Neurensin-1 plays a role in the fiber extension of the retinal neurons, as has been observed in other central nervous tissues, and indicate that the developing retina is a suitable experimental model for the analysis of Neurensin function, both in vivo and in vitro.

Neurons vigorously extend neurites during nervous tissue development and such process extension is a marked and characteristic neuronal feature that is also observed in axonal regeneration and dendritic plasticity in adulthood. Although numerous studies have revealed that several classes of proteins are involved, the precise mechanism for nerve fiber extension needs to be explored in more detail (Brady, 1991; Scott and Luo, 2001; Wong and Ghosh, 2002).
We previously isolated a novel gene, Neurensin-1/Neuro-
p24, which encodes a membrane protein with a molecular
mass of 24 kDa (Kadota et al., 1997). Based on its molecular
structure and regional distribution profiles in the brain
and cultured neurons, Neurensin-1 is specifically localized
in growing neurites but not in synaptic vesicles, and we
proposed that Neurensin-1 plays certain roles in the
transport of small vesicles to the growing distal end of
neurites in association with microtubules, thereby con-
tributing to neurite extension (Araki et al., 2002; Ida et al.,
2004). COS-7 epithelial cells extended long branching pro-
cesses when they were transfected with Neurensin-1 cDNA,
and Neurensin-1-containing small vesicles are often fused
with the plasma membrane at the growth cones. The cell
configuration altered depending on the domains deleted;
for instance, in a deletion mutant lacking the C-terminal
domain, containing a presumably organelle-targeting signal,
Neurensin-1 immunoreactive vesicles stayed at the peri-
nuclear region (probably the Golgi region), indicating that
the mutated Neurensin-1 protein was not processed at
the Golgi region to exit towards organelles. In cultured
neuroblastoma neuro2a cells, Neurensin-1 gene was up-
regulated when cells were induced to differentiate to
neurons with retinoic acid, and immunoreactive vesicles
were concentrated at the growth cones. When cells were
transfected with antisense oligonucleotide to Neurensin-1,
they transiently retracted neurites. Furthermore, in vivo
and in vitro pull-down assay confirmed Neurensin-1
binding to tubulin (Ida et al., 2004). These results strongly
suggest that Neurensin-1 plays an essential role in neurite
extension.

The neural retina is originally derived from the brain
vesicle and its developmental, structural and physiological
aspects are similar to the central nervous tissue (CNS). The
neural retina, therefore, has been considered an excellent
experimental model for the study of the CNS. This is also
the case with axonal regeneration of the retina (Aguayo,
1985). The developing retina has been intensively studied
to analyze the substantial aspects of neural development such
as cell proliferation, migration, differentiation and lineage
(Adler, 2000; Nguyen and Arnheiter, 2000). Neurite extension
is a crucial aspect of neural differentiation of retinal
neurons during regeneration and development. During
retinal development, retinal precursor cells proliferate,
migrate to the appropriate position and generate various
types of retinal cells according to a genetically programmed
timetable; in the rodent retina, the majority of ganglion and
horizontal cells appear earlier, while rods and bipolar cells
are produced later (Hinds and Hinds, 1979; Shaw and
Weber, 1983; Young, 1985). Immediately after cells are
born, they initiate neurite growth by activating a set of
genes necessary for the synthesis and transport of mem-
brane vesicles (Gordon-Weeks, 2000; Skene, 1989). Neuren-
sin-1 must be an important gene that is up-regulated for
such a process, as suggested by previous studies. In the
present study, we analyzed the localization of Neurensin-1
proteins and mRNA during the postnatal development of
the mouse retina to investigate the role of this
interesting gene in retinal development. We also examined
Neurensin-1 expression in cultured retinal cells of the
neonate mouse to determine whether it is correlated with
the in vivo developing retina. The results suggest that
Neurensin-1 plays an important role in the axonal growth
of retinal neurons, particularly in the ganglion and hori-
zontal cells.

**Localization of Neurensin-1 immunoreactivity in the postnatal
developing mouse retina.** Intense Neurensin-1 immunoreactiv-
ity was observed in the optic nerve fibers at the early stage
of postnatal development (Fig. 1). This staining in the optic
nerve fiber layer became faint as development proceeded
and disappeared in the mature retina. The optic nerve,
however, remained positively stained in later development
(Fig. 1F). The ganglion cell bodies were faintly stained and
some of the cells located at the innermost position of the
neuroblastic layer (presumably amacrine cells) were also
stained lightly on PD1 and 4 (Figs. 1A, B). Discrete cell bodies
in the outer position of the neuroblastic cell layer were
found to be positively stained on PD1 (Fig. 1A). This staining
was faint on PD1 but was obviously recognized on PD4.
These cells were identified as differentiating horizontal cells
from their position and morphology (Chien and Liem, 1995).
The inner plexiform layer was also lightly stained. On PD10,
cell bodies of presumptive horizontal cells were not stained
but many fibers were still positively stained in the outer
plexiform layer (Fig. 1C). As the retina developed further,
immunostained fibers in the outer plexiform layer gradually
disappeared and no staining could be seen on PD20, when
only faint staining was seen in the nerve fiber layer, but the
optic nerves outside the retina were still positively stained.
No reaction product was found in the outer nuclear layer
(rod cell layer).

**Localization of Neurensin-1 mRNA in the developing mouse
retina.** To identify the cell bodies that synthesize Neurensin-1,
we performed in situ hybridization with developing mouse
retinas (Fig. 2). On PD5, the ganglion cells were intensely
stained and presumptive amacrine cells at the innermost
position of the neuroblast layer were also stained (Fig. 2A).
Cell bodies located at the outer portion of the neuroblast layer
were also stained. These cells were considered horizontal cells and
they still extended several fine neurites towards the inner
layer. The whole profile of staining did not change on PD10
(Fig. 2D). On PD15, ganglion cells as well as amacrine cells were
still stained, but a little less intensely than was observed in the
previous stages. The horizontal cells were not stained
anymore. These staining profiles and the temporal changes
during postnatal development were roughly correlated with
those of immunocytochemical staining. RT-PCR also revealed
Neurensin-1 gene expression in the postnatal retinas as a
single band at a position corresponding to the mRNA size (data
not shown).

**Localization of Neurensin-1 in cultured mouse retina.** To
investigate the molecular function of Neurensin-1 in retinal
development in our further study, it is essential to examine
whether cultured mouse retinal cells also express the
Neurensin-1 gene, so we performed a cell culture study of
newborn mouse retinas. When cultured under the standard
condition, cells formed cell aggregates on an epithelial sheet
and Neurensin-1 positive processes were found within the
aggregates. These stained processes were usually short and
stayed within the aggregates (Figs. 3A, B) and gradually
disappeared as cultures proceeded. No Thy-1 immunostaining could be found in the cell aggregates. When cells were cultured under a high potassium condition, numerous cells extended long processes, which were often immunoreactive for Thy-1 (Figs. 3 C, D), suggesting that high potassium condition promotes differentiation of retinal ganglion cells (Araki et al., 1994). Neurensin-1 immunostaining showed that long fine processes were usually positively stained, which were often branching (Figs. 3E, F).

The distribution of neuronal intermediate filaments has been extensively studied in the developing mammalian retina. These filaments include neurofilament subunits, α-internexin, βIII tubulin and vimentin (Peichl and Gonzalez-Soriano, 1993; McKerracher et al., 1993; Meller et al., 1994). Low and intermediate subunits of neurofilament proteins, for instance, are localized in the ganglion cells and horizontal cells in the postnatal retinas of the mouse and rat (Shaw and Weber, 1983), while amacrine cells appear to express a different type of intermediate filament such as α-internexin (Chien and Liem, 1995).

Axon elongation is an essential aspect of neuronal differentiation and requires various molecules to be transported towards the growing distal ends in addition to the assembly of filamentous proteins. A number of proteins are involved in membrane trafficking and transport of cytoplasmic organelles. Rho-family proteins and motor proteins might provide a molecular link between membrane traffic and cytoskeletons (Cole and Lippincott-Schwartz, 1995; Murphy et al., 1996). Neurite extension requires delivery of membrane material from the Golgi apparatus (Presley et al., 1998). Neurensin-1 is a membrane protein that is considered to play a role in neurite extension by contributing to the transport of small vesicles from the Golgi region to the distal neurites (Araki et al., 2002). In the present study, we examined the distributions of Neurensin-1 protein and mRNA in the developing retina and compared the results with those previously reported on neuronal intermediate filaments.

The present results on the immunocytochemical distribution of Neurensin-1 revealed that, on PD1, intense
Neurensin-1 immunoreactivity was localized in the optic nerve fibers within the retina. As postnatal development proceeded, Neurensin-1 immunoreactivity in the optic nerve layer increased in its intensity but became faint on PD 30, when the fine-tuning for making the correct topological projections in the brain (such as the superior colliculus) has finished (Simon et al., 1994; Dallimore et al., 2002). Another interesting feature of Neurensin-1 staining was the immunoreactive profile of the horizontal cells; the distribution of Neurensin-1 was consistent with that of neurofilament protein in early postnatal development (Peichl and Gonzalez-Soriano, 1993; Chien and Liem, 1995), although Neurensin-1 staining in the horizontal cells soon disappeared after PN 15. Both GABA and its synthesizing enzyme GAD$_{67}$ are localized in the horizontal cells in a transient temporal pattern (Dkhissi et al., 2001), and it is supposed that GABA intervenes in cell maturation. These results indicate that Neurensin-1 immunoreactivity becomes faint within the retina when the retina is functionally matured. This makes a clear contrast to the distribution of structural proteins such as neural intermediate filaments that remain intensely stained in the adult retina. Neurensin-1 mRNA localization, however, remained positive in the ganglion cells and some amacrine cells in the mature retina. This indicates that these cells continue to synthesize Neurensin-1 in the mature retina and that newly synthesized protein does not stay in the cell bodies but is delivered to the distal end of neurites. The RT-PCR results also support that the expression of the Neurensin-1 gene is found in mature retinas (data not shown). These facts suggest that Neurensin-1 plays a role in maintaining neurites by supplying membranous materials. In the mouse brain, intense distribution of Neurensin-1 mRNA is found in the pyramidal cells of the cerebral cortex and cells in the nuclei of the limbic system including the hippocampus (unpublished results), where increases in the dendritic spine density and the plasticity of the dendritic pattern occur in the adult animal (Moser et al., 1994). Membrane turnover and continuous transport of membranous vesicles must occur in neurites to maintain and reconstruct neuronal fibers, particularly in the areas where active synaptic remodeling continues.
In the retinal cell culture, two types of cells were stained for Neurensin-1. It was shown previously that, under the standard culture condition for the newborn rat retina, ganglion cells do not normally differentiate, but under a high potassium condition, numerous ganglion cells differentiate and extend long neurites (Araki et al., 1994). This was also confirmed here in the retinal culture of the mouse. The present results suggest that the long stained fibers found only under a high potassium condition are from ganglion cells, while the short neurites observed in the cell aggregates of the standard culture are from amacrine or horizontal cells. The localization of Neurensin-1 in cultured retina cells and its up-regulation under certain culture conditions indicate that retinal cell culture can be used as a model experimental system to study the role of Neurensin-1 in the central nervous tissue. We have previously described the localization of Neurensin-1 in the peripheral nerves such as the dorsal root ganglion. Overexpression of the Neurensin-1 gene in the dorsal root ganglion neurons stimulates fiber elongation, while antisense oligonucleotide transfection causes retraction of neurites transiently (Ida et al., 2004). It is interesting to perform these experiments in the retinal culture and furthermore, it will be significant to determine whether target nerve tissues of retinal ganglion cells stimulate ganglion neurons to extend fibers by up-regulating Neurensin-1 gene expression.

Materials. Eyes from ICR strain mice were used in the present study. All of the experimental procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Genebank accession number of Neurensin-1/Neuro-p24 is D83206.

Immunocytochemistry. Mice of the ages of postnatal days (PD) 1, 4, 6, 7, 10, 13, 16, 20 and 30 were sacrificed by an intraperitoneal injection of excess sodium pentobarbital (20 mg/kg) (Nembutal, Boehringer Ingelheim, Australia). The eyeballs were enucleated and fixed with a mixture of 4% paraformaldehyde (PFA) and 0.3% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB) at 4 °C for 10 min, and were then immersed in 4% PFA at 4 °C overnight. In some cases, eyeballs were fixed merely with 4% PFA. Frozen sections of 20-μm thickness were cut on a cryostat, and were processed for immunocytochemistry by a free-floating method. In some cases, sections of 10-μm thickness were placed on gelatin-coated glass slides and were subsequently processed for immunocytochemistry.
Cell culture of newborn mouse retina. Retinal cells were obtained from neonatal mice eyes within 24 h after birth. The method for cell culture was fundamentally the same as described in our previous paper (Araki et al., 1994). Briefly, newborn mice were deeply anesthetized with ether and killed by decapitation, and the eyes were enucleated. The neural retina was separated cleanly from other ocular tissues, and was treated with 0.05% EDTA for 20 min at room temperature followed by incubation in 0.25% trypsin (Difco, 1:250) for 20 min at 37 °C. About 1 × 10⁶ cells (derived from one eyeball) were seeded into a 35-mm culture dish precoated with collagen. The medium was Dulbecco's modified MEM supplemented with 6% FBS (Hyclone, Utah), 55 mg/ml sodium pyruvate and 0.6% glucose.

For immunocytochemical staining with anti-Neurensin-1 and anti-Thy-1, cultures were fixed with either ice-chilled 2% PFA or a mixture of 2% PFA and 0.3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 5 min followed by a second fixation for 3 h with 2% PFA in the same buffer. Cultures were washed thoroughly with 50 mM phosphate-buffered saline (PBS, pH 7.3) and subsequently processed for immunocytochemistry with the avidin-biotin-peroxidase complex (ABC) method, as previously described. The anti-Thy-1 monoclonal antibody (OX-7) was purchased from Chemicon (Temecula, USA) and was diluted at 1:100.

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References


