Transient expression of $GABA_A$ receptor α2 and α3 subunits in differentiating cerebellar neurons

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Abstract

In the adult mammalian brain, synaptic transmission mediated by γ-amino butyric acid (GABA) plays a role in inhibition of excitatory synaptic transmission. During brain development, GABA is involved in brain morphogenesis. To clarify how GABA exerts its effect on immature neurons, we examined the expression of the $GABA_A$ receptor α2 and α3 subunits, which are abundantly expressed before α1 and α6 subunits appear, in the developing mouse cerebellum using *in situ* hybridization. Proliferating neuronal precursors in the ventricular zone and external granular layer expressed neither α2 nor α3 subunits. Hybridization signals for the α2 and α3 subunit mRNAs first appeared in the differentiating zone at embryonic day 13 (E13). The α2 subunit was detected in the migrating and differentiating granule cells and cerebellar nucleus neurons until postnatal day 14 (P14). Hybridization signals for the α3 subunit mRNA, on the other hand, were localized in the developing Purkinje cells and cerebellar nucleus neurons, and disappeared from Purkinje cells by the end of first postnatal week. Taken together, this indicated that the α2 and α3 subunits were abundantly expressed in distinct types of cerebellar neurons after completing cell proliferation while forming the neural network. These results suggest that GABA might extrasynaptically activate the $GABA_A$ receptors containing α2 and/or α3 subunits on the differentiating neurons before finishing the formation of synapses and networks, and could be involved in neuronal differentiation and maturation in the cerebellum.

Classification Terms

Theme A: Development and Regeneration

Topic: Neurotransmitter systems and channels

Key words: Purkinje cell, granule cell, cerebellar nucleus, Bergmann glia, *in situ* hybridization

1. Introduction

In the adult central nervous system (CNS), γ-amino butyric acid (GABA) is a predominant neurotransmitter, which mediates fast inhibitory synaptic transmission and regulates excitatory activity of neurons [34, 43, 45]. Recent studies have revealed that GABA serves as a excitatory transmitter during brain development and induces trophic responses, such as changes in cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation and cell death [5, 6, 7, 8, 20, 35, 45, 60, 62].

Molecular biological and morphological studies revealed that subunit compositions of the $GABA_A$ receptors drastically change during brain development [3, 12, 14, 27, 49]. Nevertheless, it has not been clearly demonstrated which types of $GABA_A$ receptors were expressed in distinct types of neurons during their ontogeny, such as cell proliferation, neuronal migration, neurite-extension and synapse-formation. To clarify how GABA exerts its effect on immature neurons, we investigated the expression of the $GABA_A$ receptor subunits in developing three types of cerebellar neurons, granule cells, Purkinje cells and cerebellar nucleus neurons, which organize the major stream of neural circuitry in the cerebellum [18, 30, 31]. The cerebellum provides an ideal system for the investigation of brain development, since it is easy to morphologically determine the development and maturation of each type of neuron [2, 19, 23, 42, 46, 50, 54]. Among more than twenty subunits composing $GABA_A$ receptors, we chose α subunits, since they may reflect the functional diversity of the $GABA_A$ receptors [34, 36, 37, 44, 55]. Furthermore, among six α subunits, we focused on the α2 and α3 subunits, which are abundantly expressed in the developing cerebellum and are thought to be involved in brain development [12, 27, 49]. We examined the developmental changes in expression of the α2 and α3 subunits in the three types of major cerebellar neurons, and tested the relationship between alterations in expression and compositions of $GABA_A$ receptors and GABAergic roles in neuronal development.

We found that Purkinje cells expressed the α3 subunit, granule cells expressed the

α2 subunit, and that the cerebellar nucleus neurons expressed both subunits, transiently during their development. Both subunits appeared after the final mitosis, and disappeared after GABAergic synapse formation. These results suggested that GABA might extrasynaptically activate the $GABA_A$ receptors containing α2 and/or α3 subunits on the differentiating neurons before finishing the formation of neural networks and might be involved not in inhibitory synaptic transmission, but in differentiation and maturation of distinct neurons in the cerebellum.

2. Materials and Methods

2.1 Animals

We examined mice from the C57BL/6CrSlc strain at embryonic day 13 (E13, mating date=E0), E15, E17, postnatal day 0 (P0), P7, P14, and P21.

2.2 In situ hybridization

The 45-mer oligonucleotide probes used for the detection of the $GABA_A$ receptor α2 and α3 subunits were 3'- TTC AGC TGG CTT GTT CTC TGG CTT CTT GTT CGG TTC TGG CGT CGT –5', which is complementary to the nucleotide residue 1216-1260 of α2, and 3'- CAG ATA AGT AGC CTT GGG TGA AGC AAT CGC TGT TGG AGT TGA AGA-5' which is complementary to nucleotide residue 1450-1494 of α3 [61].

In situ hybridization histochemistry was performed as described in a previous paper [57]. Under deep ether anesthesia, embryonic mice were taken from the uteri of the mother mice and brains were removed from the skulls of postnatal mice. Heads of embryonic mice and brains of postnatal mice were immediately frozen in powdered dry ice and cut into sagittal sections at a thickness of 20μm. After fixation with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) and pre-hybridization, hybridization was performed at 42°C for 10 hours in a hybridization buffer containing 50% formamide, 0.1M Tris-HCl (pH.7.5), 4×SSC (1×SSC; 150mM NaCl and 15mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2% sarkosyl, 250μg/ml of salmon sperm DNA, 10% dextran

sulfate, 0.1M dithiothreitol, and 10^4 dpm/μl of ^{35}S-labeled oligonucleotides. The glass slides were rinsed in 2×SSC containing 0.1% sarkosyl for 40 minutes at room temperature and in 0.1×SSC containing 0.1% sarkosyl for 80 minutes at 55°C. The sections were dipped in nuclear track emulsion (NTB2, Kodak) and exposed for two months.

To confirm the results using the above probes, we used additional non-overlapping oligonucleotide probes. 3'- GAC AGG ATC TTT GGA AAG ATT CGG GGC ATA GTT GGC AAC AGC TAC –5' complementary to the nucleotide residue 1147-1191 of the α2, and 3'- CTT GGC CAG ATT GAT AGG ATA GGT GGT ACC CAC TAT GTT GAA GGT –5' complementary to the nucleotide residue 1357-1401 of the α3 [61]. Identical expression-patterns were obtained with additional oligonucleotide probes (data not shown). The specificity of each oligonucleotide was also verified by a control hybridization experiment in the presence of a 20-fold excess amount of each unlabeled oligonucleotide. The specific signals for the α2 and α3 subunits were abolished in the presence of a 20-fold excess amount of respective unlabeled oligonucleotides used for the detection of the α2 and α3 subunit mRNAs, respectively (data not shown).

These experiments were permitted by The Animal Care and Use Committee of Hokkaido University School of Medicine.

3. Results

3.1 $GABA_A$ receptor α2 and α3 subunits in the mouse cerebellum at P21

Hybridization signals for the α2 subunit were detected in the Purkinje cell layer (Pu), white matter (WM) and cerebellar nuclei (Fig. 1A). Weak signals for the α3 subunit, on the other hand, were only localized in the cerebellar nucleus (Fig. 1B). A higher magnification bright field micrograph revealed that silver grains for the α2 subunit accumulated in the Purkinje cell layer (arrowheads), but were negative in the Purkinje cell bodies (Fig. 1C). In contrast, no significant signals for the α3 subunit were detected in the cerebellar cortex (Fig.

1D). In the cerebellar nuclei, silver grains for both α2 (Fig. 1E) and α3 (Fig. 1F) subunits accumulated at the cerebellar nucleus neurons (arrows). These results demonstrated that Purkinje cells and granule cells did not expressed either α2 or α3 subunits, while cerebellar nucleus neurons faintly expressed both subunits. In addition, Bergmann glia might have contained the α2 subunit. The expression patterns of the α2 and α3 subunits were identical to those in adult rats reported previously.

3.2 Developmental changes in expression of the $GABA_A$ receptor α2 subunit

Hybridization signals for the α2 subunit first appeared in the dorsal surface of the cerebellum (arrowheads and arrows) at embryonic day 13 (E13), but were negative in the ventricular zone where neuronal precursors were proliferating (Fig. 2A, B). The signals accumulated at the cerebellar nuclei (Nu) and inner half of the external granular layer (arrowheads and black arrows) at E15 (fig. 2C, D). Furthermore, weak signals were detected in the differentiating zone (white arrow, Fig. 2C). Subsequently, α2-signals at the inner half of the external granular layer (arrowheads) expanded their distribution towards the posterior cerebellum, and surrounded the cerebellum at E17 (Fig. 2E). At P0 (Fig. 2F), two bands of signals were clearly discernible in the cortex (arrowheads). Higher magnification bright field micrographs (Fig. 2G) revealed that silver grains were arrayed at the inner half of the external granular layer (arrows) and under the Purkinje cell layer (crossed arrows and asterisks). In the cerebellar nuclei, silver grains densely accumulated at the large neurons (Fig. 2H). During the formation of the internal granular layer, dense α2-signals were detected both in the external and internal granular layers (Fig. 3A-D). Within the external granular layer at P7 (Fig. 3B), the signals accumulated at the deep half, where granule cells migrate tangentially and wait for radial migration [21, 50, 51, 54]. At P14 (Fig. 3D), silver grains localized entirely in the thin external granular layer, where the proliferation of granule cell precursors had finished. In the molecular layer, a few silver grains were sometimes detected at the migrating granule cells (arrowheads, Fig. 3B, D), but were absent in the Purkinje cell bodies. In the internal granular

layer, the intensity of the signals for the α2 subunit decreased after P7 (Fig. 1C, Fig. 3B, C). In the cerebellar nuclei, silver grains were confined to the cell bodies of large neurons at P7 (Fig. 3E) and P14 (Fig. 3F), but the density decreased during development. These results demonstrated that the α2 subunit was transiently and abundantly expressed in differentiating and maturating granule cells and cerebellar nucleus neurons.

3.3 Developmental changes in expression of the $GABA_A$ receptor α3 subunit

Hybridization signals for the α3 subunit mRNA first appeared in the dorsal part of the cerebellum (arrowheads) at E13 (Fig. 4A), and were widely detected in the cerebellum (arrowheads) at E15 (Fig. 4B). On the higher magnification bright field micrographs, silver grains for the α3 subunit mRNA were localized at the differentiating zone (arrows) at E13 (Fig. 4C) and the primitive cerebellar nuclei (Nu) at E15 (Fig. 4D). Furthermore, α3-signals were sparsely and weakly localized under the differentiating Purkinje cell layer (arrows, in Fig. 4D), where Purkinje cells and other interneurons migrate. The α3- signals, however, were negative in the ventricular zone and external granular layer at E15 (Fig. 4D). Subsequently, α3-signals were confined to the differentiating Purkinje cell layer (arrowheads) and cerebellar nuclei at E17 (Fig. 4E) and P0 (Fig. 4F-H). Silver grains for the α3 subunit were localized at the Purkinje cell layer (arrows, Fig. 4G) and accumulated to cerebellar nucleus neurons (arrows, Fig. 4H). At P7, faint signals transiently and weakly appeared in the internal granular layer (Fig. 4I, 4J), but disappeared from the Purkinje cell bodies (Fig. 4J). α3-signals were only detected in the cerebellar nuclei at P14 (Fig. 4K). These results demonstrated that the α3 subunit was transiently and densely expressed at differentiating and maturating Purkinje cells and cerebellar nucleus neurons.

4. Discussion

In the present study, we examined the developmental changes in expression of the $GABA_A$ receptor α2 and α3 subunits in the three major cerebellar neurons, Purkinje cells,

granule cells, and cerebellar nucleus neurons. Purkinje cells transiently expressed the α3 subunit, granule cells expressed the α2 subunit, and the cerebellar nucleus neurons expressed both subunits, while they were differentiating and maturating. The α1 and α6 subunits, on the other hand, appear during postnatal development and are abundantly expressed in the adult cerebellum [3, 14, 27, 49]. Taken together, developmental changes in expression of the $GABA_A$ receptor α subunits in the cerebellar neurons are summarized in Table 1.

4.1 GABAergic roles during brain development

GABA appears long before the onset of synaptogenesis and acts as a trophic factor for differentiating neurons [11, 24, 25, 28, 29, 35, 59, 60]. Before synaptogenesis, GABA may extrasynaptically released via plasma membrane [16], activate GABA receptors on neighboring neurons, mediate depolarized membrane potential, elevate cytosolic calcium and play roles in CNS development, such as (1) regulation of cell proliferation, (2) cell migration, and (3) neuronal maturation, including synaptogenesis [5, 6, 7, 8, 10, 35, 45]. This study demonstrated the early expression of the $GABA_A$ receptor α2 and α3 subunits in differentiating and maturating cerebellar neurons, supporting the above results of physiological and cell-biological investigations.

GABA acts as an anti-proliferation molecule [7, 32], and the GABA receptors abundantly expressed in postmitotic neurons of the ventricular zone [6, 32, 33]. This study demonstrated that proliferating cells in the ventricular zone adjacent to the fourth ventricle expressed neither α2 nor α3 subunits, while both subunits were abundantly detected in the differentiating zone at E13, when progenitors of Purkinje cells and cerebellar nucleus neurons were massively generating [39, 63]. In the external granular layer, on the other hand, the α2 subunit continued to be detected in the deeper half until P14. Granule cells were born in the external granular layer beneath the pia matter. After the final mitosis, granule cells are translocated into the deeper half of the external granular layer, the premigratory zone, extend horizontal processes and migrate tangentially [21, 22, 42, 54]. Taken together, cerebellar

neurons start to express the α2 and α3 subunits when neuronal proliferation finishes, suggesting that the onset of expression of these $GABA_A$ receptor subunits might stop cell proliferation and start cell differentiation in the cerebellum.

Exposure of neurons to GABA or GABA agonist enhanced the growth rate of neuronal processes, and facilitated synapse formation [1, 4, 40, 56, 62], suggesting that activation of $GABA_A$ receptor might lead to neuronal maturation. Furthermore, GABA also induces the expression of GABA receptor subunits, which mediate inhibitory synaptic transmission [1, 7, 9, 15, 38, 41]. Here, we demonstrated that the α2 and α3 subunits were abundantly expressed in cerebellar neurons, after settling at the final destination, until the end of synaptogenesis. Taken together, the above suggests that GABA might be involved in neuronal maturation by activating the $GABA_A$ receptors containing α2 and α3 subunits.

4.2 Relationship between GABAergic roles in the developing cerebellum and subunit-compositions of the $GABA_A$ receptors

$GABA_A$ receptor α2 and α3 subunits continued to be expressed in the cerebellar neurons during the differentiation and maturation period. After finishing the network formation, in contrast, the expression of both subunits decreased, and the α1 and α6 subunits were dominantly expressed in the adult cerebellum [14, 27, 58]. These results suggested that neuronal development might be mediated by the $GABA_A$ receptors containing the α2 and α3 subunits, and the GABAergic inhibitory transmission is mediated by the receptors including the α1 and α6 subunits. Although it is generally considered that the changes in GABAergic roles may be reflected by the Cl^- reversal potential [10, 45, 47, 52, 53], the above suggested that the subunit-composition might partially related to the expression of roles.

4. 2 Regulatory mechanism underlying the changes in $GABA_A$ receptor subunit composition

Previous studies and this one revealed that Purkinje cells start to express the α1 subunit instead of the α3 subunit during the first postnatal week, and granule cells start to

express the α1 and α6 subunits instead of the α2 subunit during the second and third postnatal week [3, 27]. When the pattern of subunit-expression changes, both types of neurons formed excitatory and inhibitory synapses coincidentally. These results suggested that changes in subunit composition might be induced by synapse-formation. In a previous study, we examined expression of the $GABA_A$ receptor α subunits under aberrant conditions of the reeler cerebellum [57]. Although neuronal maturation of malpositioned Purkinje cells was assumed to be arrested due to lack of excitatory input from parallel fibers and the presence of climbing fibers' multiple innervation, expression of the α3 subunit was almost as negative as that in the normal cerebellum, and malpositioned Purkinje cells abundantly expressed the α1 subunit [13, 57]. These results indicated that formation of excitatory synapses with parallel fibers is independent of decreasing expression of the α3 subunits, and that formation of GABAergic synapse with Golgi cells and neighbor Purkinje cells might be crucial for changes in subunit composition. In conclusion, changes in subunit composition during cerebellar development might be induced by GABAergic synapse formation.

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Figure Legends

Figure 1 Expression of the $GABA_A$ receptor α2 and α3 subunits in the mouse cerebellum at P21

(A and B) Dark field micrographs showing the expression of α2 (A) and α3 (B) subunits in the cerebellum.

(C and D) Bright field micrographs showing the expression of the α2 (C) and α3 (D) subunits in the cerebellar cortex consisting of the molecular (Mo), Purkinje cell (Pu) and granular (Gr) layers. Silver grains for the α2 subunit (arrowheads) accumulated between the Purkinje cell bodies (asterisks) (C).

(E and F) Bright field micrographs showing the expression of α2 (E) and α3 (F) subunits in the cerebellar nucleus (Nu). Silver grains for the α2 (E) and α3 (F) accumulated at the large neurons (arrows).

Figure 2 Developmental changes in expression of the $GABA_A$ receptor α2 subunit in the cerebellum at E13 (A, B), E15 (C, D), E17 (E), and P0 (F-H)

(A and B) Dark field (A) and bright field (B) micrographs showing the expression of the α2 subunit in the cerebellum at E13. The signals were localized at the dorsal surface of the cerebellum (arrowheads and arrows), but were negative in the ventricular zone (VZ).

(C and D) Dark field (C) and bright field (D) micrographs showing the expression of the α2 subunit in the cerebellum at E15. The signals (arrowheads, arrows) were localized at the bottom of the external granular layer (EGr) and developing nucleus (Nu). Furthermore, weak signals were detected under the Purkinje cell layer (white arrow).

(E) A dark field micrograph showing the expression of α2 subunit in the cerebellum at E17. The signals were localized at the surface of the cerebellum (arrowheads), sub-cortical region (white arrows), and nucleus (Nu).

(F-H) Dark field (F) and bright field (G, H) micrographs showing the expression of the α2 subunit in the cerebellum at P0. The signals (arrowheads, arrows) were localized at the bottom of the external granular layer (EGr), and under the Purkinje cell layer (Pu, crossed

arrows), but were negative in the Purkinje cell layer (Pu, F, G). In the nucleus, silver grains accumulated at the large neurons (arrows, H).

Figure 3 Developmental changes in expression of the $GABA_A$ receptor α2 subunit in the cerebellum at P7 (A, B, E) and P14 (C, D, F)
(A and B) Dark field (A) and bright field (B) micrographs showing the expression of the α2 subunit in the cerebellum at P7. Hybridization signals were localized at the deep part of the external granular layer (EGr, arrows), migrating granule cells (arrowheads) in the molecular layer (Mo), and internal granular layer (IGr), but were negative at the Purkinje cell bodies (asterisks).
(C and D) Dark field (C) and bright field (D) micrographs showing the expression of α2 subunit in the cerebellum at P14. Hybridization signals were localized in the external granular layer (EGr), migrating granule cells (arrowheads) in the molecular layer (Mo), and internal granular layer (IGr). In addition, silver grains often accumulated between Purkinje cell bodies (arrows).
(E and F) Bright field micrographsshowing the expression of α2 subunit in the cerebellar nuclei at P7 (E) and P14 (F). Silver grains accumulated at the large neurons (arrows).

Figure 4 Developmental changes in expression of the $GABA_A$ receptor α3 subunit in the cerebellum at E13 (A, C), E15 (B, D), E17 (E), P0 (F-H), P7 (I, J), and P14 (K)
(A and B) Dark field micrographs showing the expression of α3 subunit in the cerebellum at E13 (A) and E15 (B). Hybridization signals were detected at the dorsal part of the cerebellum (arrowheads) and developing nucleus (Nu).
(C and D) Bright field micrographs showing the expression of the α3 subunit in the cerebellum at E13 (C) and E15 (D). Silver grains were localized in the differentiating zone (DZ), nucleus (Nu), and differentiating Purkinje cells (arrows in D), but were negative in the ventricular zone (VZ) and external granular layer (EGr).

(E and F) Dark field micrographs showing the expression of α3 subunit in the cerebellum at E17 (E) and P0 (F). Hybridization signals were detected at the cerebellar surface (arrowheads) and nucleus (Nu).

(G and H) Bright field micrographs showing the expression of α3 subunit in the cerebellar cortex (G) and nucleus (H) at P0. Silver grains were localized in the Purkinje cell layer (arrows, Pu) and accumulated at the nucleus neurons (arrows in H), but were negative in the external granular layer (EGr).

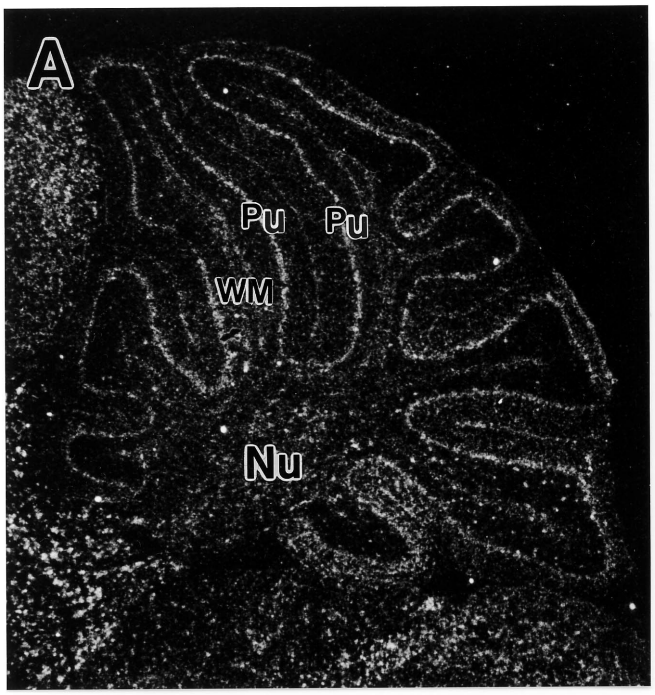
(I and J) Dark field (I) and bright field (J) micrographs showing the expression of α3 subunit in the cerebellum at P7. In the cortex, faint signals were detected in the internal granular layer (IGr), but external granular layer (EGr) and Purkinje cells (asterisks) were negative.

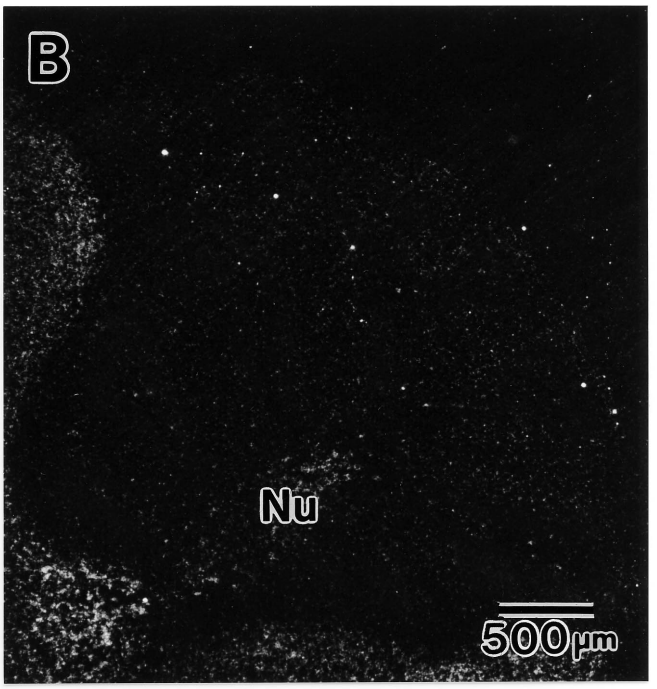
(K) Dark field micrograph showing the expression of α3 subunit in the cerebellum at P14. Faint signals were only detected in the cerebellar nucleus (Nu).

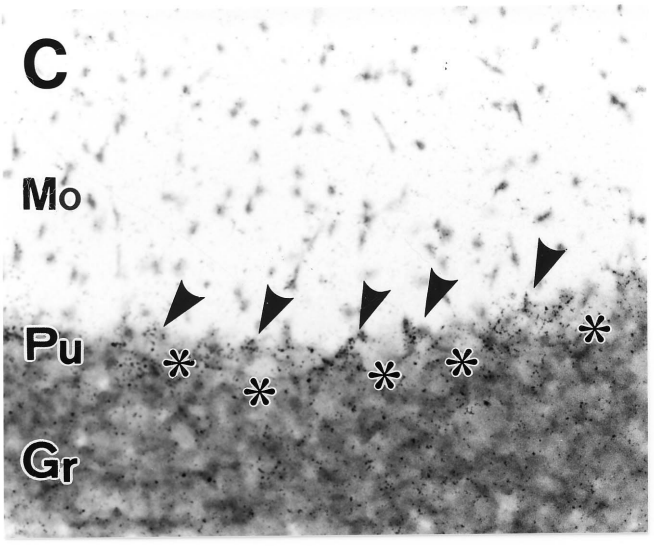
Table 1

Changes in expression of the predominant α subunits in the cerebellar cells

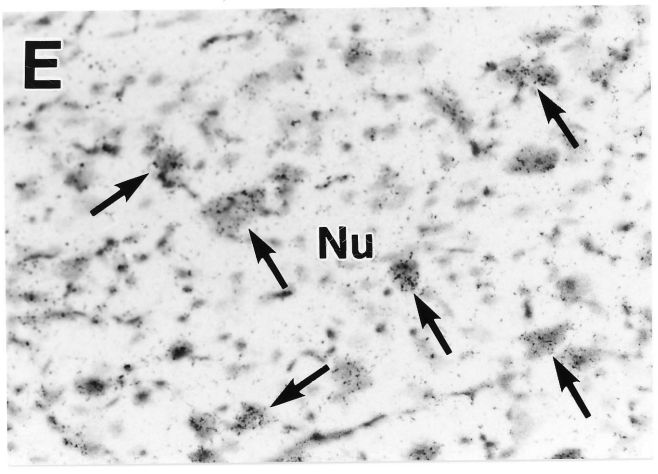
	Proliferating stage	Migrating and Differentiating stage	Matured stage
Purkinje cells	negative	α3 subunit	α1 subunit
Granule cells	negative	α2 subunit	α1 and α6 subunits
Nucleus neurons	negative	α2 and α3 subunits	α1 subunits
Bergmann glia	negative	α2 subunit	α2 subunits

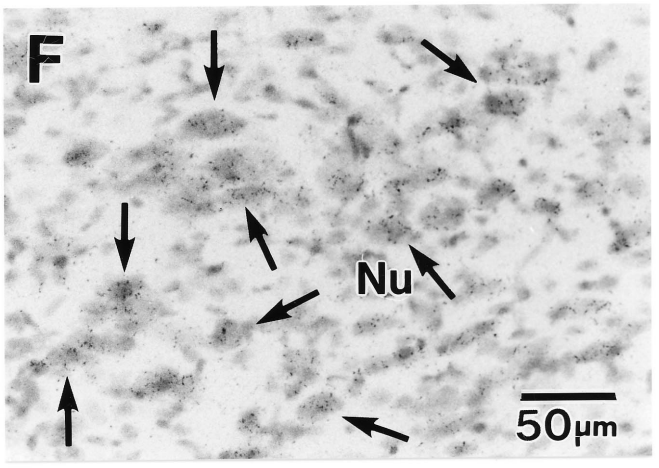

A
Pu
Pu
WM
Nu

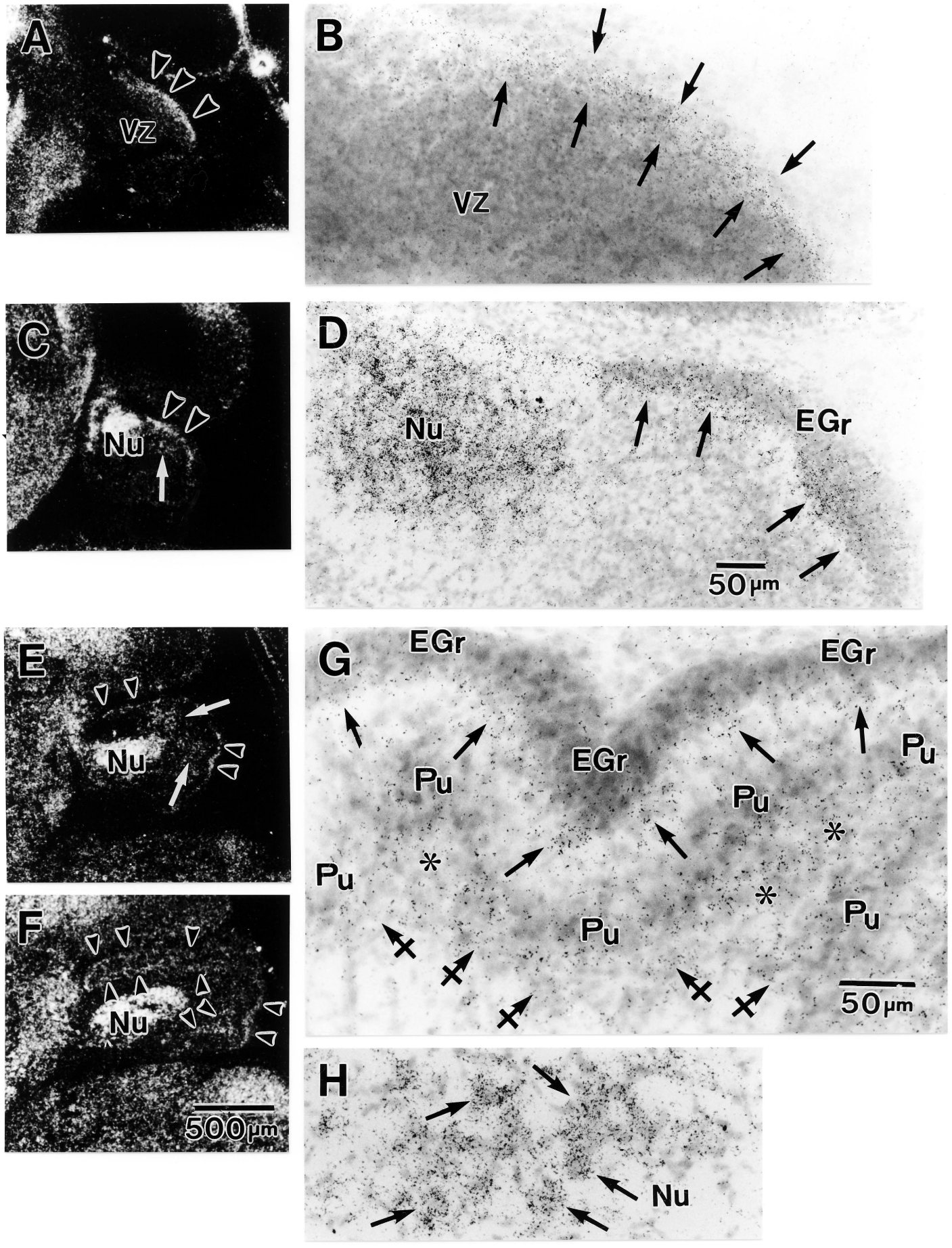

B
Nu
500μm

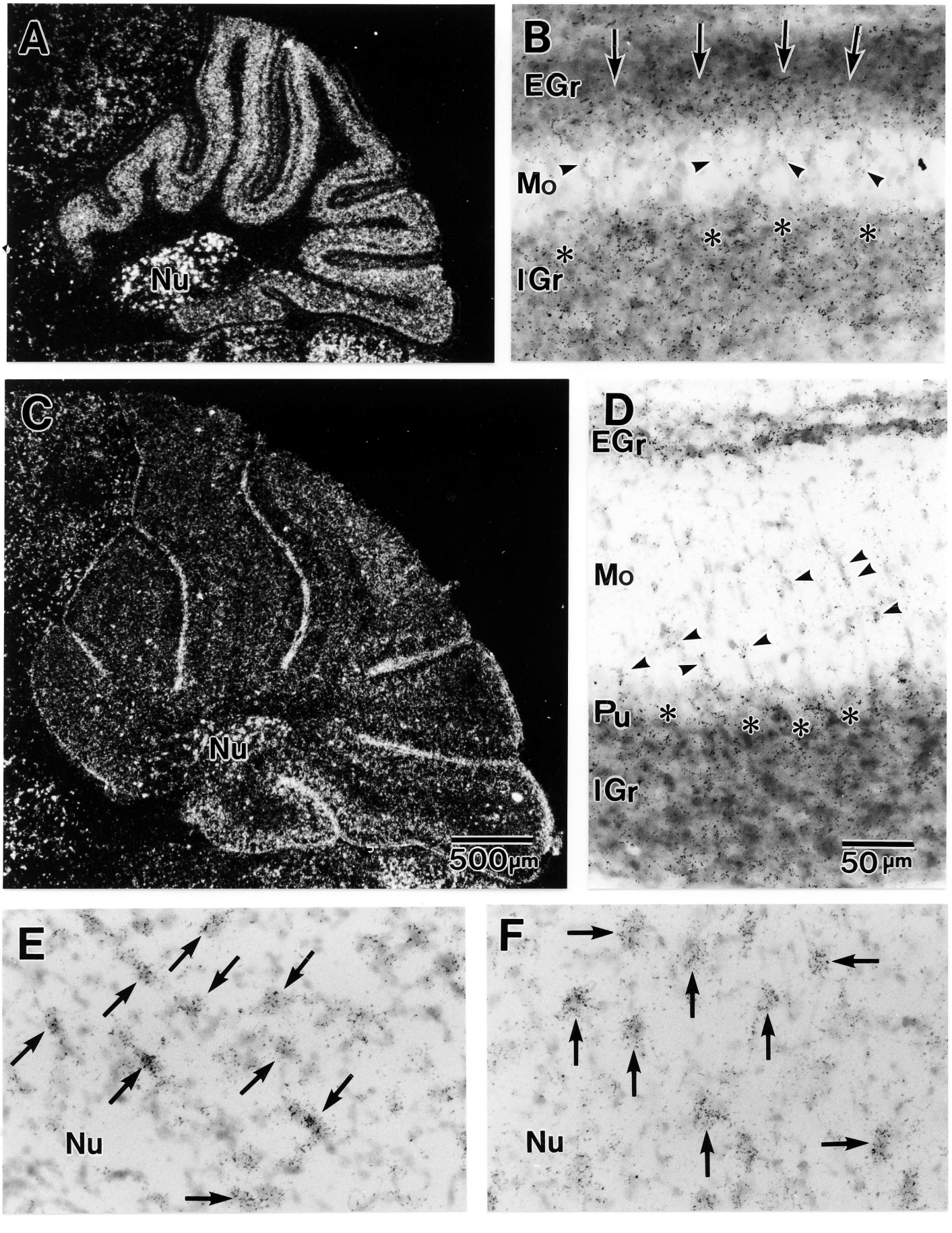

C
Mo
Pu
Gr

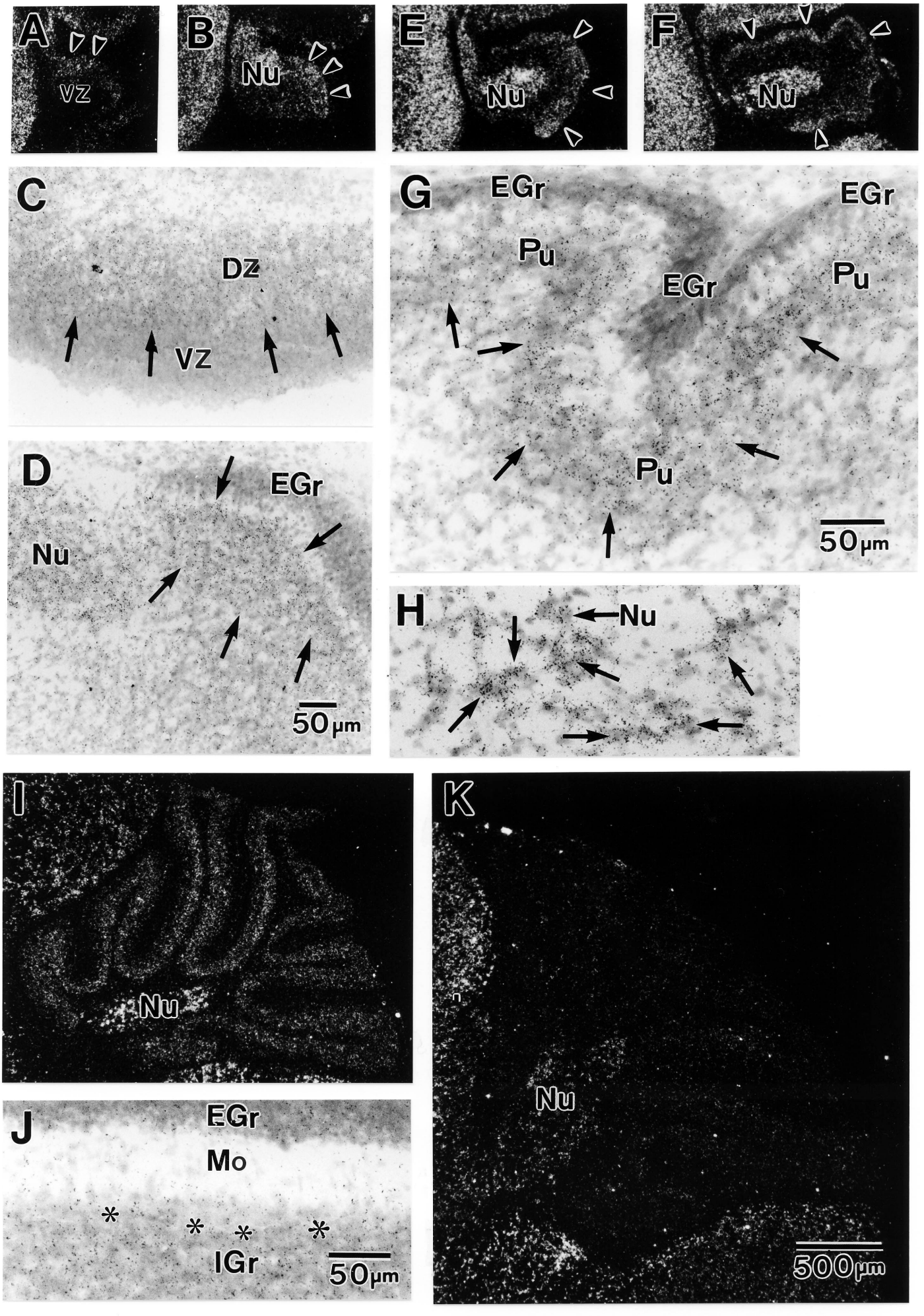

D
Mo
Pu
Gr


E
Nu


F
Nu
50μm


A
VZ
B
VZ
C
Nu
D
Nu
EGr
50μm
E
Nu
F
Nu
500μm
G
EGr
EGr
EGr
Pu
Pu
Pu
Pu
Pu
Pu
50μm
H
Nu


A
Nu
B
EGr
Mo
IGr
C
Nu
500μm
D
EGr
Mo
Pu
IGr
50 μm
E
Nu
F
Nu


A
vz
B
Nu
E
Nu
F
Nu
C
Dz
vz
G
EGr
Pu
EGr
EGr
Pu
Pu
50μm
D
EGr
Nu
50μm
H
Nu
I
Nu
J
EGr
Mo
IGr
50μm
K
Nu
500μm