Original Paper

Developmental Neuroscience

Dev Neurosci 2003;25:332–342
DOI: 10.1159/000073510

Received: December 2, 2002
Accepted: May 5, 2003

Expression of Glutamate Transporters in the Medial and Lateral Vestibular Nuclei during Rat Postnatal Development

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Key Words
Vestibular nuclei · Excitatory amino acid transporters · Postnatal development, rat

Abstract
The postnatal developmental expression and the distribution of the glutamate transporters (GLAST, GLT-1 and EAAC1) were analyzed in rat vestibular nuclei (VN), at birth and during the following 4 weeks. Analyses were performed using reverse transcriptase-polymerase chain reaction and immunoblotting of GLAST, GLT-1 and EAAC1 mRNA and protein during the postnatal development of the VN neurons and their afferent connections. We also studied the distribution of each glutamate transporter in the medial and lateral VN by use of immunocytochemistry and confocal microscopy. GLAST, GLT-1 and EAAC1 mRNA and protein were present in the VN at each developmental stage. GLAST was highly expressed mainly in glia from birth to the adult stage, its distribution pattern was heterogeneous depending on the region of the medial and lateral VN. GLT-1 expression increased dramatically during the second and third postnatal weeks. At least during the first postnatal week, GLT-1 was expressed in the soma of neurons. EAAC1 was detected in neurons and decreased from the third week. These temporal and regional patterns of GLAST, GLT-1 and EAAC1 suggest that they play different roles in the maturation of glutamatergic synaptic transmission in the medial and lateral VN during postnatal development.

Introduction

The vestibular nuclei (VN) are the first relay of primary vestibular neurons. They are located dorsally in the brainstem and constitute a complex of 4 major different nuclei: the medial (MVN), the lateral (LVN), the superior and the descending VN [Gerrits, 1990; Büttner-Ennever, 1992; Rubertone et al., 1995]. Each nucleus can be subdivided further based primarily on cytological criteria or differences in afferent or efferent projections [Brodal, 1974]. Bordering the fourth ventricle, the MVN contains various types of cells; it is divided into a parvocellular region (pMVN) and in a magnocellular region (mMVN). This region is characterized by a massive arrival of vestibular primary afferents and endings [Walberg et al., 1958; Gacek, 1969; Schwarz et al., 1977]. The LVN contains the giant Deiters' cells which are concentrated in the dorsal part of the LVN (dLVN). This subdivision receives a mas-

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sive inhibitory input from the cerebellar cortex [Angaut and Brodal, 1967; de Zeeuw and Berrebi, 1995].

Glutamate has been demonstrated to be the principal neurotransmitter involved in synaptic transmission between the primary and the second-order vestibular neurons [Raymond et al., 1984; Doi et al., 1990; de Waele et al., 1995; Smith and Darlington, 1997]. The afferent vestibular terminals arrive at their target before birth and mature during the first month of life [Morris et al., 1988; Ashwell and Zhang, 1998; Maklad and Fritzsch, 2002]. Data obtained from structural analyses [Karhunen, 1973; Puyal et al., 2002] and electrophysiological studies [Lannou et al., 1979; Johnston and Dutia, 1996; Murphy and Du Lac, 2001] suggest that maturation of the VN are coordinated with the remodeling of their afferent connections. The differential expression of glutamate receptors during this period has been shown to be related to postsynaptic regulation, associated with the synaptic maturation of the vestibular neuron connections [Sans et al., 2000].

Glutamatergic signaling plays an important role in the regulation, organization and development of neuronal circuits. As a result, the control of extracellular glutamate concentration is essential for synaptic transmission and maturation. The extracellular glutamate concentration is controlled by nonvesicular glial and neuronal transporting proteins that remove glutamate from the synaptic cleft and neighboring synapses [for a review, see Seal and Amara, 1999; Tanaka, 2000; Danbolt, 2001]. Five subtypes of excitatory amino acid transporters (EAATs) have so far been cloned and identified in the rat brain: GLAST (EAAT1) [Storck et al., 1992, Tanaka, 1993] and GLT-1 (EAAT2) [Pines et al., 1992] are localized primarily in astrocytes; EAAC1 (EAAT3) [Kanai and Hediger, 1992] is widely distributed in neurons; EAAT4 [Fairman et al., 1995] is localized mainly in cerebellar Purkinje cells, and EAAT5 is localized in the retina [Arriza et al., 1997]. The levels of GLAST and GLT-1 are low in the immature rat brain and increase as the rat matures. Conversely, the levels of EAAC1 are higher in the neonatal brain than in the adult brain [Furuta et al., 1997; Sims and Robinson, 1999].

Due to the critical role that glutamate neurotransmission plays in the development of the VN circuitry, we investigated whether the expression of GLAST, GLT-1 and EAAC1 is developmentally regulated during the maturation of the vestibular neuronal circuits in the rat VN. We used reverse transcriptase-polymerase chain reaction (RT-PCR) to identify the three EAAT mRNAs, and Western blotting to analyze protein levels. We used immunocytochemistry to compare the spatial and cellular distributions of the transporters in the MVN and LVN at birth and during the first postnatal month. The neuronal localization of each glutamate transporter was determined by double-labeling with antineurofilament (anti-NF) antibodies.

Materials and Methods

Animals and Tissue Preparation

Experiments were performed on Sprague-Dawley rats at different stages between birth [postnatal day 0 (P0)] and the adult stage (P28). Animals were cared for in accordance with the guidelines of the French 'Ministère de l'Agriculture et de la Forêt' (authorization number 34-134) and the European Community Council directive of November 24, 1986 (86/609/EEC). The animals were anesthetized and decapitated. For immunocytochemical analyses in older animals, anesthetized animals were perfused intracardially.

RT-PCR Analysis

For the RT-PCR and Western blot analyses, microdissection of the VN was performed as described previously in detail [Puyal et al., 2002]. The expression of GLAST, GLT-1 and EAAC1 mRNAs in VN was investigated at the following postnatal stages: P0, P4, P7, P14, P21 and P28 (n = 6). Total RNA was isolated and purified from rat VN using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. The purified mRNA (1 μg) was then reverse transcribed using SuperScript II Reverse Transcriptase (10 U/μl; Invitrogen, France) to produce cDNA. The 20 μl reaction mixture contained: 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 1 U/μl RNase inhibitor, 12.5 ng/μl oligo $(dT)_{12-18}$, 10 m*M* 1,4-dithiothreitol and 0.5 m*M* deoxynucleotide triphosphates. The RT mixture was incubated at 50°C for 50 min, then the enzyme was inactivated by heating at 70°C for 15 min (Thermal cycler, Hybaid). The negative RT control was performed by omitting SuperScript II.

For each transporter, the resulting cDNA was then amplified by PCR. The 50 μl reaction mixture contained: 4 μl of single-strand DNA preparation, 5 μl 10 × PCR buffer (Gibco, BRL), 0.2 m*M* deoxynucleotide triphosphates, 0.75 m*M* $MgCl_2$ and 0.03 U/μl *Taq* DNA polymerase (Invitrogen). Specific upstream and downstream primers were used (0.5 pmol/μl). Forty cycles were programmed, each cycle consisted of a denaturation step (94°C for 1 min), a primer annealing step (52°C for GLAST, 55°C for GLT-1 and EAAC1 each for 1 min) and an extension step (72°C for 1½ min). This was followed by a final elongation at 72°C for 15 min in a PCR system (Thermal cycler, Hybaid).

The specific primers (table 1) used to detect GLAST, GLT-1 and EAAC1 mRNA in the rat VN were designed based on the sequences published by Li et al. [1994] and synthesized by Eurogentec. The positive control consisted of adult rat hippocampal or cerebellar extracts, which contain the three different EAATs. The negative PCR control consisted of omitting the cDNA.

Antibodies

Antibodies directed against EAATs were a kind gift from Prof. Niels Christian Danbolt (University of Oslo, Anatomical Institute, Blindern, Oslo, Norway). GLAST, GLT-1 and EAAC1 antibodies were prepared as described previously [Lehre et al., 1995; Lehre and Danbolt, 1998].

Table 1. GLAST, GLT-1 and EAAC1 specific primers

EAATs	Forward primer 3′	Reverse primer 5′
GLAST	5′-CTGTCTGCCACGGGTTTCTC-3′	5′-GAGATTGGTAGCGGTGATAA-3′
GLT- 1	5′-GGTGACAGGCAAAGTTCCAG-3′	5′-TCTCATCATTTCCAGTCTCA-3′
EAAC1	5′-TGTGACGCTGATAGTGATGA-3′	5′-GACAGATTCTGGTGGATTTC-3′

1 Anti-GLAST: anti-A522 (amino acid sequence 522–541: PYQLIAQDNE PEKPVADSET), rabbit 68468.
2 0nti-GLT-1: anti-B12 (amino acid sequence 12–26: KQVEVRMHDS HLSSE), rabbit 26970.
3 EAAC-1: anti-C491 (amino acid sequence 491–524: TLDNEDSDTK KSYINGGFAVDKSDTISFTQ TSQF), rabbit 7DO993.

Double-labeling was performed with a monoclonal mouse anti-NF, 200 kD (N52, Sigma-Aldrich, St. Louis, Mo., USA).

Western Blot

The VN were microdissected from rats on postnatal days P0, P2, P4, P7, P14, P21 and P28. Tissues were homogenized in protein lysis buffer containing 2 m*M* ethylenediaminetetraacetic acid, 10% glycerol, 2.3% sodium dodecyl sulfate (SDS), 62 m*M* Tris-HCl pH 6.8 and protease inhibitor cocktail (Boehringer Mannheim). The resulting lysates were boiled for 5 min and centrifuged. The protein concentrations were determined by a BCA Protein Reagent (Pierce, Rockford, Ill., USA). Equivalent amounts of proteins (10 µg for GLAST and GLT-1 and 20 µg for EAAC1) were separated by 7.5% SDS-polyacrylamide gel electrophoresis and then electroblotted onto a nitrocellulose membrane (Hybond ECL, Boerhringer Mannheim). Transfers were performed overnight in 20 m*M* Tris, 150 m*M* glycine, 20% methanol buffer, and 0.01% SDS. The membranes were incubated for 1 h in a blocking solution consisting of 8% nonfat dried milk in Tris-buffered saline (TBS; 50 m*M* Tris, 150 m*M* NaCl, pH 7.4) at room temperature. The membranes were probed with antibodies directed against EAATs: anti-GLAST (A522, 0.2 µg/ml), anti-GLT-1 (B12, 0.2 µg/ml) and anti-EAAC1 (C491, 0.25 µg/ml), diluted in 5% bovine serum albumin (Sigma Aldrich) in TBS for 1 h at room temperature. Immunoblots were washed three times for 5 min each in TTBS, 0.2% Tween 20 (Sigma Aldrich) in TBS and then incubated for 1 h at room temperature with peroxidase-conjugated antirabbit immunoglobulin G (IgG, 1:750; Jackson-ImmunoResearch, Laboratories, West Grove, Pa., USA). The immunoreactive bands were detected by exposure to film using an enhanced chemiluminescence kit (ECL, Amersham, Little Chelfont, UK). Molecular sizes were estimated by separating prestained molecular weight markers (219–8.8 kD) in parallel (Kaleidoscope Prestained Standards, Bio-Rad Laboratories, Calif., USA). The marker proteins included bovine serum albumin (67 kD), which was of a similar size to each of the glutamate transporters studied. To quantify the protein bands of interest, the film was digitally scanned and analyzed by densitometry. For each transporter, the densitometric analyses of immunoreactive bands were carried out at each developmental stage and expressed as mean ± SEM of the optical density and as percentage of the maximum value. The comparison between the mean values at different stage of development was calculated by Student's t test. Proteins were isolated from at least 3 rats for each stage. Adult rat hippocampus protein extract was used as a positive control for each EAAT.

Immunocytochemistry and Confocal Microscopy

The younger rats (P0, P2, P4) were killed by decapitation and fixed by immersion in a fixative solution [4% paraformaldehyde in 0.1 *M* phosphate-buffered saline (PBS) pH 7.4]. The older animals (P7, P14, P21, P28) were deeply anesthetized with Nembutal (60 mg/kg injected intraperitoneally) and transcardially perfused with the same cold fixative solution. The brainstem and cerebellum were rapidly removed and postfixed overnight in the same fixative. The specimens were then incubated overnight in 30% sucrose in PBS before sectioning on a cryostat. Frozen coronal sections (14 µm) of the brainstem were collected on polylysine-coated slides. Sections were preincubated in PBS containing 10% donkey serum and 0.3% Triton X-100 for 1 h. The samples were then incubated overnight with the primary antibodies at room temperature. Double-labeling was carried out by simultaneously incubating samples with anti-GLAST (0.2 µg/ml), anti-GLT-1 (0.2 µg/ml), or anti-EAAC1 (0.5 µg/ml) plus a monoclonal mouse anti-NF (200 kD, diluted at 1:700). Serial sections were stained with GLAST, GLT-1 or EAAC1 antibodies. The sections were rinsed in PBS, three times for 10 min, and then incubated with secondary antibodies (Jackson ImmunoResearch Laboratories), a mixture of fluorescein isothiocyanate-conjugated donkey antirabbit IgG and Texas Red-conjugated donkey antimouse IgG, each diluted 1:200, for 1 h 30 min. After rinsing in PBS, the sections were mounted using FluorSave (Calbiochem, San Diego, Calif., USA). Control experiments consisted in omitting the primary antibodies; no staining was observed under these conditions. A positive control for GLAST, GLT-1 and EAAC1 immunoreactivity was performed using cerebellum sections. Immunofluorescence was analyzed by confocal laser microscopy on a Biorad MRC-1024 system (Axiovert 100TV, Zeiss) equipped with a ×20 or a ×40 oil immersion objective. Confocal images were displayed as single optical sections. The two types of fluorescence in double-labeling experiments were sequentially visualized on the same section, using two distinct filters. Images were acquired in a separate mode. Images were then processed with Adobe Photoshop 4.0. We observed sections of the brainstem from 3–5 rats at each postnatal stage.

Results

RT-PCR Analysis

GLAST, GLT-1 and EAAC1 mRNA were detected in VN at birth and at each developmental stage from P0 to P28. PCR using specific primers revealed products of the

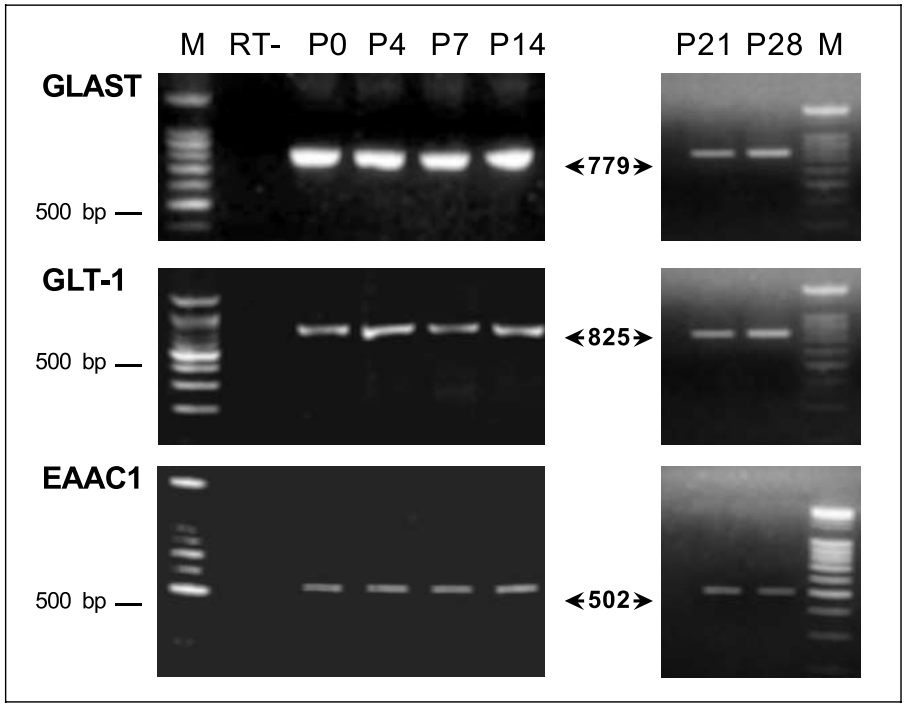

Fig. 1. RT-PCR analysis for GLAST, GLT-1 and EAAC mRNA in VN from birth (P0) to P28. The sizes of the EAAT bands were: 779 bp for GLAST, 825 bp for GLT-1 and 502 bp for EAAC1. RT = Negative control; M = molecular weight marker, 100 bp DNA ladder.

expected size: 779 bp for GLAST, 825 bp for GLT-1 and 502 bp for EAAC1 (fig. 1). No bands were detected in the negative PCR control.

Western Blot Analysis

Western blots were used to compare the levels of GLAST, GLT-1 and EAAC1 proteins at each developmental stage and showed that the 3 EAATs were present in VN at birth and at all postnatal stages (fig. 2). The GLAST band was detected at around 67 kD, which is consistent with the expected size of the protein. The amount of GLAST detected remained relatively high and constant at all postnatal stages (fig. 2A). From birth, GLT-1 was detected as a band of around 67 kD, corresponding to the predicted protein size. The densitometric analysis indicated that the level of this protein gradually increased from birth to P14. GLT-1 levels drastically increased after P14, peaking at P21 and P28 (fig. 2B). From P14, the immunoblots presented a wide band that extended from 67 to 220 kD. EAAC1 immunoblots showed a specific band just below 67 kD, in accordance with the predicted size of the protein. Less important bands were observed during the first week, they decreased and disappeared progressively. EAAC1 levels remained stable from birth to P14, before decreasing at the end of the third week (fig. 2C).

Immunocytochemical Analysis

The distribution of each EAAT was analyzed at each developmental stage on rat coronal sections in mMVN and dLVN (fig. 3A) at the level of the vestibular nerve root [Paxinos and Watson, 1986] by double-labeling with an antibody directed against GLAST, GLT-1 or EAAC1 plus an antibody directed against NF, a specific neuronal marker (fig. 3).

Distribution of GLAST in Medial and Lateral VN

At birth, the density of GLAST was greatest in the region corresponding to the mMVN. In the dLVN, where the giant neurons were NF-labeled, the density of GLAST was very low (fig. 3B, 4A). Vestibular root fibers arriving in the VN were visualized by NF labeling (fig. 3B). During postnatal VN development, the neuron somas and their neurite processes increased in size, but the pattern of GLAST distribution (a high-density labeled network around neuron somas) did not change during the maturation of the MVN (fig. 3B, D). In the dLVN, GLAST was rare at birth (fig. 3B, 4A). However, the density of GLAST labeling around the giant neurons increased during the second postnatal week (fig. 3C, 4B). Double-labeling showed that GLAST was never colocalized with neurons or fibers labeled with NF (fig. 3B, C, D).

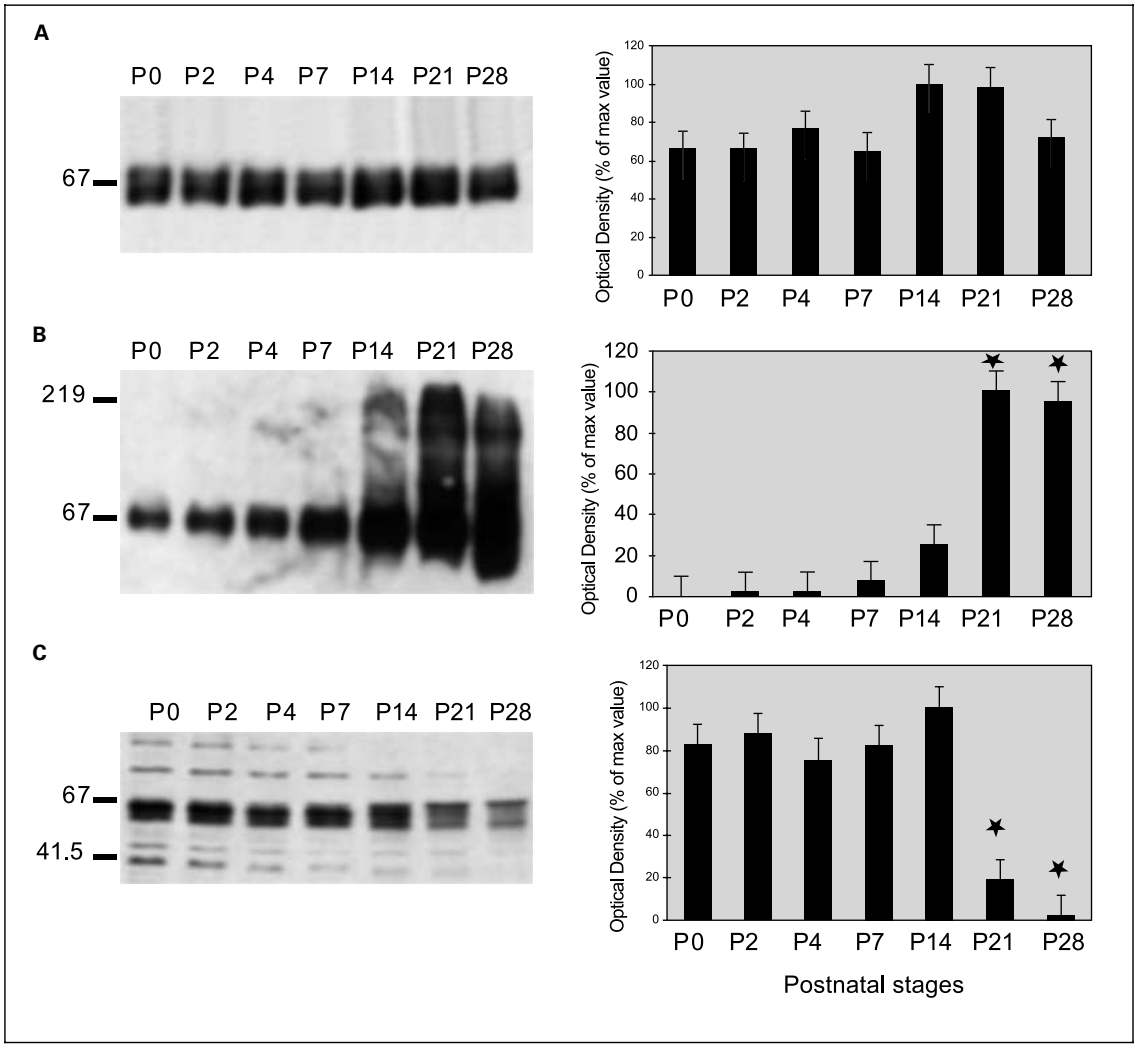

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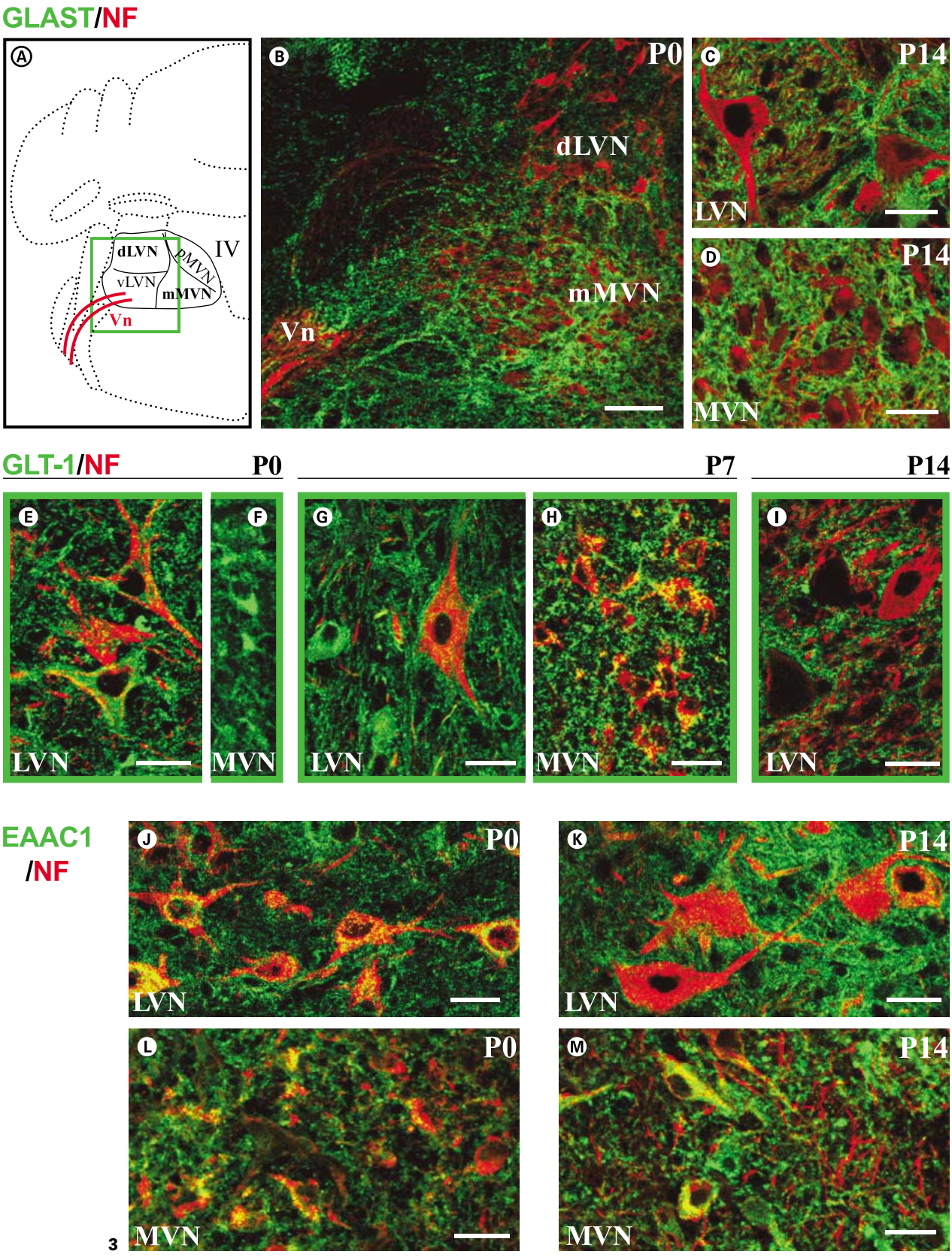
Fig. 2. Western blot analyses for GLAST (**A**), GLT-1 (**B**) and EAAC1 (**C**) in VN from birth (P0) to P28. The membranes were immunostained with anti-GLAST (0.2 µg/ml), anti-GLT-1 (0.2 µg/ml) or anti-EAAC1 (0.5 µg/ml). The blots shown are representative of three independent experiments. Molecular mass markers (in kD) are indicated on the left. The histograms show densitometric analyses of immunoreactive protein levels detected at each stage. Results are expressed as the percentage of the maximum optical density value ± SEM. There was a significant difference (Student's t test; $p < 0.005$) between the values at P21 and P28 and the values at earlier stages for GLT-1 (stars) and EAAC1 (stars).

Fig. 3. Distribution of immunoreactivities for GLAST, GLT-1 and EAAC1 (green) in the LVN and MVN during postnatal development. Double-labeling with NF (red). **A** Schematic representation of subdivisions of the LVN and MVN on adult coronal section of the brainstem at the level of vestibular nerve: vLVN = ventral LVN; IV = fourth ventricle; Vn = vestibular nerve in red. The green frame delimits the 3B confocal image. Distribution of immunoreactivity for GLAST (green). **B** At birth, in the mMVN, GLAST labeling appeared as a dense network around the NF-labeled somas (red). GLAST labeling was nearly absent in the dLVN. Notice the fibers of the vestibular root nerve (Vn) and the giant neurons of the LVN labeled with NF (red). **C** At P14, in the dLVN, GLAST (green) was not evenly distributed, mainly around the giant neurons. **D** At P14, in the mMVN, the distribution pattern of GLAST was similar to that at birth. GLAST immunoreactivity (green) was never localized in neurons labeled with NF (red). **E**–**I** Distribution of GLT-1 (green). At P0, in the dLVN (**E**) and in the mMVN (**F**), single GLT-1 labeling; at P7, in the dLVN (**G**) and in the mMVN (**H**), GLT-1 immunoreactivity was present in the soma of neurons colocalized (yellow) with NF (red). From P14, there is no GLT-1 labeling in the neuronal soma as illustrated in the dLVN (**I**). **J**–**M** Distribution of EAAC1 (green). **J**, **L** At birth, in the dLVN (**J**) and in the mMVN (**L**), EAAC1 immunoreactive puncta (green) were observed in several neuronal soma, colocalized (yellow) with NF labeling (red). **K** EAAC1 immunolabeling was also scattered around the neuronal soma. At later stages of postnatal development, in the dLVN, the density of EAAC1 immunoreactivity decreased in the soma and increased around them as illustrated at P14. The labeling is not evenly distributed. **M** In the mMVN, EAAC1 labeling appeared as sparse puncta. Bar: 100 µm in **B**; 25 µm in **C**–**M**.



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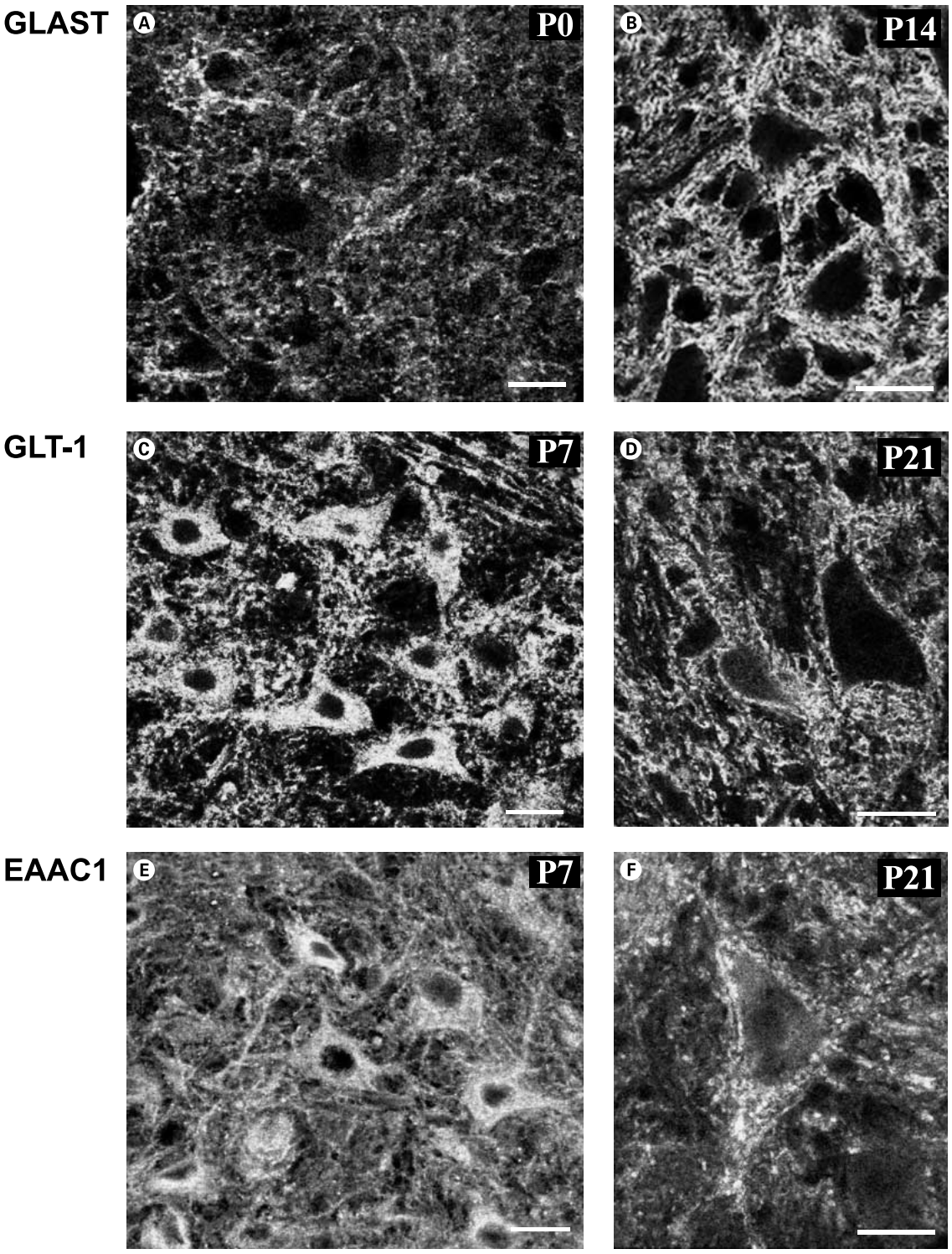

Fig. 4. Comparison of the distributions of GLAST, GLT-1 and EAAC1 in the dLVN. **A**, **B** Changes in GLAST immunoreactivity at P0 and P14; notice the increased density of the label between the unlabeled somas. **C** GLT-1 labeling was distributed as immunopositive puncta around and in the neuron somas at P7. **D** GLT-1 immunoreactivy could not be detected in the neuronal somas at P21. The GLT-1 labeling (**D**) between the somas was less dense than GLAST labeling. **E** Strong EAAC1 labeling was observed in the soma of giant neurons at P7. **F** At P21, the EAAC1 immunoreactivity was light in the somas and distributed around them as patches of immunoreactive puncta. Bar is 25 μ*M* in **A**–**F**.

Distribution of GLT-1 in Medial and Lateral VN

At birth and during the first postnatal week, GLT-1 was detected in the dLVN (fig. 3E) and mMVN (fig. 3F). GLT-1 immunoreactivity was mainly restricted to some neuron somas, although a lightly labeled network was visible around these somas. The neuronal distribution of GLT-1 was clearly observed in the soma of neurons at P7 (fig. 3G, H, 4C). This somatic distribution in the dLVN and mMVN was no longer observed from P14 onwards (fig. 3I, 4D). From the second week onwards, the density of the labeling between the somas increased lightly (fig. 3I, 4D). Due to the absence of neuronal somatic labeling, the distribution of GLT-1 presented some similarities with that of GLAST (fig. 4B).

Distribution of EAAC1 in Medial and Lateral VN

At birth, EAAC1 was detected in and around neurons in MVN and LVN. Double-labeling with EAAC1 and NF showed a colocalization in neuron soma at birth (fig. 3J, L). In MVN, the pattern of EAAC1 distribution was similar during postnatal development, as illustrated at P14 (fig. 4L). From this stage, in the LVN, EAAC1 immunoreactivity decreased in the large neuron soma and increased in patches situated between the somas at P14 (fig. 3K), and labeling was more localized around the soma at P21 (fig. 4F). The pattern of EAAC1 (fig. 4F) was different from those of GLAST (fig. 4B) and GLT-1 (fig. 4D).

Discussion

EAAT in VN

During the development of the central nervous system, glutamate modulates the growth of neuronal processes, synapse formation and synapse elimination [Rabacchi et al., 1992]. Thus, by controlling extracellular glutamate concentration, EAATs play an important role in the development of neuronal connections. Previous studies on glutamate uptake in the rat brain have shown that glutamate uptake is low at birth and increases to a maximum level in the adult [Collard et al., 1993]. This increase occurs during the active period of synaptogenesis, in correlation with an intense vesicular uptake activity [Christensen and Fonnum, 1992]. Several authors have studied the expression of EAAT mRNA and protein in the central nervous system during development [Shibata et al., 1996; Sutherland et al., 1996; Furuta et al., 1997; Ullensvag et al., 1997; Sims and Robinson, 1999]. However, no investigations have looked at EAATs in the MVN and LVN in relation to the development of vestibular function.

Our study demonstrated that the three glutamate transporters (GLAST, GLT-1 and EAAC1) are present in the VN from birth until the adult stage. The RT-PCR and immunoblot results revealed bands of the expected molecular sizes [Li et al., 1994; Lehre et al., 1995; Lehre and Danbolt, 1998]. Immunoblot densitometric analysis showed three different expression patterns. The levels of GLAST remained relatively constant throughout development, the levels of GLT-1 increased dramatically from the third postnatal week, whereas the levels of EAAC1 decreased from the third postnatal week.

During development of the VN, different aspects of structural and electrophysiological maturation of neurons and connections have been observed [Karhunen, 1973; Lannou et al, 1979; Johnston and Dutia, 1996; Murphy and Du Lac, 2001; Puyal et al., 2002]. The synaptic maturation of the vestibular glutamatergic connection is associated with a differential expression of ionotropic glutamate receptors in relation to a postsynaptic regulation [Sans et al., 2000]. The specific distribution patterns of the three glutamate transporters is also consistent with their involvement in the maturation of the glutamatergic synaptic transmission in the VN development.

Changes in the Levels and the Distribution of GLAST during Postnatal Medial and Lateral VN Development

In the mouse brain, the levels of GLAST peak at P14 in diverse brain regions in relation to the development and structural maturation of glutamatergic neuronal connections [Shibata et al., 1996]. Conversely, in the rat VN, immunoblot analysis showed that the level of GLAST did not significantly vary during postnatal development. The level of GLAST was as high at birth and in early stages of development, as in the later stages and in adult rats. This difference may be due to the early establishment of the vestibular afferents in the VN before birth [Morris et al., 1988]. However, immunocytochemical analysis indicated that the distribution differed between the mMVN and the dLVN. The density of GLAST labeling was high at all stages in the mMVN and low in the dLVN at early stages. A previous electron microscopy study had shown in the adult rat that the mMVN receives mainly terminals of vestibular afferent neuronal fibers [Schwarz et al., 1977], whereas the dLVN is devoid of these direct inputs [Brodal, 1974].

In the dLVN, the differential distribution of GLAST during postnatal development could be related to the later maturation of some nonvestibular afferents [Karhunen, 1973]. This part of the VN undergoes major remodeling of its connections after the first postnatal week due to the

arrival and maturation of cerebellar connections [Bäurle and Grüsser-Cornehls, 1994; Puyal, 2002]. Thus, the high level of GLAST immunoreactivity in the MVN seems to be related with the release of glutamate as a neurotransmitter by vestibular nerve endings [Demêmes et al., 1984; Raymond et al., 1984], which are the major synaptic input in this area [Brodal, 1974; Schwarz et al., 1977].

This suggests that GLAST has a major effect on the vestibular excitatory synaptic transmission in the target areas from the earliest stages of postnatal development.

The GLAST distribution pattern and the absence of colocalization with neuronal marker NF indicate that in the VN, GLAST is preferentially distributed in glia as in the brain and in the cerebellar cortex [Lehre et al., 1995; Ullensvag et al., 1997]. The presence of GLAST in glia is related to the control of glutamate receptor activation, and to glutamine synthesis, which is in turn involved in neuronal energy metabolism [Sonnewald et al., 1997; Magistretti and Pellerin, 1999; Stanimirovic et al., 1999].

Changes in the Levels and Distribution of GLT-1 during Postnatal Medial and Lateral VN Development

In the VN, GLT-1 levels increased after the second postnatal week. In the brain, the levels of GLT-1 increased steeply from P7 onwards in a number of different structures [Shibata et al., 1996; Furuta et al., 1997]. The maximal level is reached at P14 in the mouse brain, particularly in the hippocampus, cerebral cortex, septum and striatum [Shibata et al., 1996]. In the cerebellum, the maximal levels of GLT-1 are reached after the third postnatal week [Ullensvag et al., 1997; Danbolt, 2001]. Typically, GLT1 and GLAST are expressed in mature glia, where they contribute to protect the brain from neuronal damage resulting from the excessive release of glutamate into the extracellular space [Tanaka et al., 1997; Wang et al., 1998; Namura et al., 2002].

The increase in GLT-1 levels revealed by densitometric analysis of the immunoblots on P21 corresponds to the mature stages of development of the VN. The broad bands that appeared on the immunoblots from P14 have already been described and may correspond to glycoproteins at different stages of posttranslational modification or multimers [Furuta et al., 1997; Plachez et al., 2000; Danbolt, 2001; Guillet et al., 2002].

In the VN, the levels of GLT-1, but not those of GLAST, increased in the later stages of postnatal maturation. GLT-1 and GLAST have been shown to be differentially regulated in different areas of the brain [Lehre et al., 1995; Ullensvag et al., 1997]. The aspects of VN maturation that are related to these changes are unclear, but a few studies have looked at the late morphological and functional aspects of maturation: (1) the vestibular nerve endings acquire the mature termination pattern on their precise target areas after the second postnatal week [Maklad and Frisch, 2002; Puyal et al., 2002], and (2) the discharge of vestibular neurons gradually increases over the first postnatal month to reach the adult level [Lannou et al., 1979; Johnston and Dutia, 1996; Murphy and Du Lac, 2001]. It remains to be determined whether GLT-1 has a specific function related with the functional development of the VN network. GLT-1 may be involved in fine modulation and may be regulated by neuronal activity.

The neuronal distribution of GLT-1 from birth to P7 is noteworthy. Transient GLT-1 neuronal expression has been described during early development in the growing axons of the mouse spinal cord before glial expression in astrocytes [Yamada et al., 1998]. During ovine brain development, GLT-1 is transiently expressed in neurons and may play a role in the topographical organization of the cerebellar cortex at the middle of the gestation period [Northington et al., 1998, 1999]. Transient neuronal expression of GLT-1 has also been observed in cultures of rat hippocampal neurons from embryonic day 17 [Brooks-Kayal et al., 1998; Plachez et al., 2000], or during the early postnatal stages [Mennerick et al., 1998]. In the adult, GLT-1 neuronal expression has only been described in sensory systems such as the rat and monkey retina, where GLT-1 is present in cones and cone bipolar cells [Rauen and Kanner, 1994; Rauen, 2000].

After the first week, GLT-1 was not colocalized with the neuronal marker, suggesting that it displays a classical glial distribution. However, we cannot rule out the possibility that some GLT-1 is present in distal dendrites. The cellular changes coincide with the dramatic increase in protein expression from the second week.

In the VN, the two glial transporters, GLT-1 and GLAST, may be present in distinct glial populations as in hippocampal cocultures [Perego et al., 2000]. The identification of different types of glial cells in the VN during development opens complex questions. Indeed, in the adult VN, glial fibrillary acidic protein identifies mature astrocytes [de Waele et al., 1996], but at early stages of VN development, this marker is not detected [pers. obs.]. Further studies, using other glial markers, will be necessary to identify the various glial populations and their maturation during postnatal development.

Changes in the Levels and Distribution of EAAC1 during Postnatal Medial and Lateral VN Development

Immunoblot analysis indicated that the level of EAAC1 decreased after the second postnatal week. The higher level of protein at immature stages is in accordance with previous studies on the central nervous system, which showed that EAAC1 levels are higher in the newborn brain than in the adult brain [Furuta et al., 1997]. This suggests that EAAC1 plays an important role during the early stages of postnatal development of the VN neurons.

During VN development, EAAC1 expression in the neurons corresponds to the distribution described in the central nervous system [Danbolt, 2001]. Moreover, in the LVN, at birth and during the first postnatal week, EAAC1 expression was very high in the soma, whereas after the second postnatal week, EAAC1 levels decreased in the soma and increased in the neuritic network around the soma. This redistribution coincides with the massive development of dendritic processes in the VN neurons [Karhunen, 1973; Puyal et al., 2002]. Recent studies have shown that EAAC1 is located in the peripheral zone of the postsynaptic area [He et al., 2000, 2001] and it has been suggested that the postsynaptic neuronal glutamate transporter, EAAC1, could contribute to reduce the activation of synaptic or extrasynaptic glutamate receptors and to limit the activation of N-methyl-*D*-aspartate by controlling neurotransmitter spillover [Diamond, 2001]. Our observations of the redistribution of EAAC1 labeling after the second week suggest that the protein is targeted from the soma to the dendritic spines in the mature vestibular network.

Conclusion

The differential GLAST, GLT-1 and EAAC1 distributions observed during postnatal development of VN suggest that each transporter has a different role. EAAC1 could be important in the very early stages and GLAST and GLT-1 could play different roles in the maturation of glutamatergic synaptic transmission during postnatal development. As modifications of different ionotropic glutamate receptor subunits are involved in plasticity processes [Sans et al., 2000], expression changes of glutamate transporters may also contribute to glutamatergic synapse maturation that occur during VN postnatal development.

Acknowledgments

The authors express their great thanks to Danielle Demêmes for her critical reading of the manuscript.

References

Angaut P, Brodal A (1967): The projection of the 'vestibulocerebellum' onto the vestibular nuclei in the cat. Arch Ital Biol 105:441–479.

Arriza JL, Eliasof S, Kavanaugh MP, Amara S (1997): Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci USA 94: 4155–4160.

Ashwell KWS, Zhang L-I (1998): Prenatal development of the vestibular ganglion and vestibulocerebellar fibres in the rat. Anat Embryol (Berl) 198:149–161.

Bäurle J, Grüsser-Cornehls U (1994): Calbindin D-28k in the lateral vestibular nucleus of mutant mice as a tool to reveal Purkinje cell plasticity. Neurosci Lett 167:85–88.

Brodal A (1974): Anatomy of the vestibular nuclei and their connections; in Kornhuber HH (ed): Handbook of Sensory Physiology. Berlin, Springer, pp 240–251.

Brooks-Kayal AR, Munir M, Jin H, Robinson MB (1998): The glutamate transporter, GLT-1, is expressed in cultured hippocampal neurons. Neurochem Int 33:95–100.

Büttner-Ennever JA (1992): Patterns of connectivity in the vestibular nuclei. Ann NY Acad Sci 656:363–378.

Christensen H, Fonnum F (1992): The ontogeny of the uptake systems for glutamate, GABA, and glycine in synaptic vesicles isolated from rat brain. Neurochem Res 17:457–462.

Collard KJ, Edwards R, Liu Y (1993): Changes in synaptosomal glutamate release during postnatal development in the rat hippocampus and cortex. Brain Res Dev Brain Res 71:37–43.

Danbolt NC (2001): Glutamate uptake. Prog Neurobiol 65:1–105.

Demêmes D, Raymond J, Sans A (1984): Selective retrograde labeling of neurons in the cat vestibular ganglion with ^{3}H *D*-aspartate. Brain Res 304:188–191.

Diamond JS (2001): Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. J Neurosci 21:8328–8338.

Doi K, Tsumoto T, Matsunaga T (1990): Actions of excitatory amino acid antagonists on synaptic inputs to the rat medial vestibular nucleus: an electrophysiological study in vitro. Exp Brain Res 82:254–262.

Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG (1995): An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. Nature 375: 599–603.

Furuta A, Rothstein JD, Martin LJ (1997): Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J Neurosci 17:8363–8375.

Gacek R (1969): The course and central termination of neurons supplying vestibular end organs in the cat. Acta Otolaryngol Suppl 254:1–66.

Gerrits NM (1990): Vestibular nuclear complex; in Paxinos G (ed): The Human Nervous System. San Diego, Academic Press, pp 863–888.

Guillet B, Lortet S, Masmejean F, Samuel D, Nieoullon A, Pisano P (2002): Developmental expression and activity of high affinity glutamate transporters in rat cortical primary cultures. Neurochem Int 40:661–671.

He Y, Hof PR, Janssen WG, Rothstein JD, Morrison JH (2001): Differential synaptic localization of GluR2 and EAAC1 in the macaque monkey entorhinal cortex: A postembedding immunogold study. Neurosci Lett 311:161–164.

He Y, Janssen WG, Rothstein JD, Morrison JH (2000): Differential synaptic localization of the glutamate transporter EAAC1 and glutamate receptor subunit GluR2 in the rat hippocampus. J Comp Neurol 418:255–269.

Johnston AR, Dutia MB (1996): Postnatal development of spontaneous tonic activity in mouse medial vestibular nucleus neurones. Neurosci Lett 219:17–20.

Kanai Y, Hediger MA (1992): Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360:467–471.

Karhunen E (1973): Postnatal development of the lateral vestibular nucleus (Deiter's nucleus) of the rat. A light and electron microscopic study. Acta Otolaryngol Suppl 313:1–87.

Lannou J, Precht W, Cazin L (1979): The postnatal development of functional properties of central vestibular neurons in the rat. Brain Res 175: 219–232.

Lehre KP, Danbolt NC (1998): The number of glutamate transporter subtype molecules at glutamatergic synapses: Chemical and stereological quantification in young adult rat brain. J Neurosci 18:8751–8757.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995): Differential expression of two glial glutamate transporters in the rat brain: Quantitative and immunocytochemical observations. J Neurosci 15:1835–1853.

Li H-S, Niedzielski AS, Beisel KW, Hiel H, Wenthold RJ, Morley B (1994): Identification of a glutamate/aspartate transporter in the rat cochlea. Hear Res 78:235–242.

Magistretti PJ, Pellerin L (1999): Astrocytes couple synaptic activity to glucose utilization in brain. News Physiol Sci 14:177–182.

Maklad A, Fritzsch B (2002): The developmental segregation of posterior crista and saccular vestibular fibers in mice: A carbocyanine tracer study using confocal microscopy. Brain Res Dev Brain Res 135:1–17.

Mennerick S, Dhond RP, Benz A, Xu W, Rothstein JD, Danbolt NC, Isenberg KE, Zorumski CF (1998): Neuronal expression of glutamate transporter GLT-1 in hippocampal microcultures. J Neurosci 18:4490–4499.

Morris RJ, Beech Jn, Heinzmann CW (1988): Two distinct phases and mechanisms of axonal growth show by primary vestibular fibers in the brain demonstrated by parvalbumin immunohistochemistry. Neuroscience 27:571–596.

Murphy GJ, Du Lac S (2001): Postnatal development of spike generation in rat medial vestibular nucleus neurons. J Neurophysiol 85:1899–1906.

Namura S, Maeno H, Takami S, Jiang X-F, Kamichi S, Wada K, Nagata I (2002): Inhibition of glial glutamate transporter GLT-1 augments brain edema after transient focal cerebral ischemia in mice. Neurosci Lett 324:117–120.

Northington FJ, Traystman RJ, Koehler RC, Martin LJ (1999): GLT1, glial glutamate transporter, is transiently expressed in neurons and develops astrocyte specificity only after midgestation in the ovine fetal brain. J Neurobiol 39: 515–526.

Northington FJ, Traystman RJ, Koehler RC, Rothstein JD, Martin LJ (1998): Regional and cellular expression of glial (GLT-1) and neuronal (EAAC1) glutamate transporter proteins in ovine fetal brain. Neuroscience 85:1183–1194.

Paxinos G, Watson C (1986): The Rat Brain in Stereotaxic Coordinates. Sydney, Academic Press.

Perego C, Vanoni C, Bossi M, Massari S, Basudev H, Longhi R, Pietrini G (2000): The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal cocultures. J Neurochem 75: 1076–1084.

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992): Cloning and expression of a rat brain *L*-glutamate transporter. Nature 360:464–467.

Plachez C, Danbolt NC, Recassens M (2000): Transient expression of the glial glutamate transporters GLAST and GLT in hippocampal neurons in primary culture. J Neurosci Res 59: 587–593.

Puyal J, Devau G, Venteo S, Sans N, Raymond J (2002): Calcium-binding proteins map the postnatal development of rat vestibular nuclei and their vestibular and cerebellar projections. J Comp Neurol 451:374–391.

Rabacchi S, Bailly Y, Delhaye-Bouchaud N, Mariani J (1992): Involvement of the N-methyl-*D*-aspartate (NMDA) receptor in synapse elimination during cerebellar development. Science 256:1823–1825.

Rauen T (2000): Diversity of glutamate transporter expression and function in the mammalian retina. Amino Acids 19:53–62.

Rauen T, Kanner BI (1994): Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinae. Neurosci Lett 169:137–140.

Raymond J, Nieoullon A, Demêmes D, Sans A (1984): Evidence for glutamate as a neurotransmitter in the cat vestibular nerve. Exp Brain Res 56:523–531.

Rubertone JA, Mehler WR, Voogd J (1995): The vestibular nuclear complex; in Paxinos G (ed): The Rat Nervous System. San Diego, Academic Press, pp 773–796.

Sans NA, Montcouquiol ME, Raymond J (2000): Postnatal developmenal changes in AMPA and NMDA receptors in the rat vestibular nuclei. Brain Res Dev Brain Res 123:41–52.

Schwarz DWF, Schwarz IE, Fredrickson JM (1977): Fine structure of the medial and descending vestibular nuclei in normal rats and after unilateral transection of the vestibular nerve. Acta Otolaryngol 84:80–90.

Seal RP, Amara SG (1999): Excitatory amino acid transporters: A family in flux. Annu Rev Pharmacol Toxicol 39:431–456.

Shibata T, Watanabe M, Tanaka K, Wada K, Inoue Y (1996): Dynamic changes in expression of glutamate transporter mRNAs in developing brain. Neuroreport 7:705–709.

Sims KD, Robinson MB (1999): Expression patterns and regulation of glutamate transporters in the developing and adult nervous system. Crit Rev Neurobiol 13:169–197.

Smith PF, Darlington CL (1997): The contribution of N-methyl-*D*-aspartate receptors to lesion-induced plasticity in the vestibular nucleus. Prog Neurobiol 53:517–531.

Sonnewald U, Westergaard N, Schousboe A (1997): Glutamate transport and metabolism in astrocytes. Glia 21:56–63.

Stanimirovic DB, Ball R, Small DL, Muruganadam A (1999): Developmental regulation of glutamate transporters and glutamine synthase activity in astrocyte cultures differentiated in vitro. Int J Dev Neurosci 17:173–184.

Storck T, Schulte S, Hofmann K, Stoffel W (1992): Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci USA 89:10955–10959.

Sutherland ML, Delaney TA, Noebels JL (1996): Glutamate transporter mRNA expression in proliferative zones of developing and adult murine CNS. J Neurosci 16:2191–2207.

Tanaka K (1993) Cloning and expression of a glutamate transporter from mouse brain. Neurosci Lett 159:183–186.

Tanaka K (2000): Functions of glutamate transporters in the brain. Neurosci Res 37:15–19.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K (1997): Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276:1699–1702.

Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1997): Differential developmental expression of two rat brain glutamate transporter proteins GLAST and GLT. Eur J Neurosci 9: 1646–1655.

de Waele C, Campos Torres A, Josset P, Vidal PP (1996): Evidence for reactive astrocytes in rat vestibular and cochlear nuclei following unilateral inner ear lesion. Eur J Neurosci 8:2006–2018.

de Waele C, Mühlethaler M, Vidal PP (1995): Neurochemistry of central vestibular pathways. Brain Res Brain Res Rev 20:24–46.

Walberg F, Bowsher D, Brodal A (1958): The termination of the primary vestibular fibers in the vestibular nuclei in the cat. An experimental study with silver methods. J Comp Neurol 110: 391–419.

Wang GJ, Chung HJ, Schnuer J, Lea E, Robinson MB, Potthoff WK, Aizenman E, Rosenberg PA (1998): Dihydrokainate-sensitive neuronal glutamate transport is required for protection of rat cortical neurons in culture against synaptically released glutamate. Eur J Neurosci 10: 2523–2531.

Yamada K, Watanab M, Shibata T, Nagashima M, Tanaka K, Inoue Y (1998): Glutamate transporter GLT-1 is transiently localized on growing axons of the mouse spinal cord before establishing astrocytic expression. J Neurosci 18:5706–5713.

de Zeeuw CI, Berrebi AS (1995): Postsynaptic targets of Purkinje cell terminals in the cerebellar and vestibular nuclei of the rat. Eur J Neurosci 7:2322–2333.