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Immunohistochemical study of the astrocytes of the adult rat cochlear nuclei

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SUMMARY

Astrocytes play crucial roles in the organization, function and maintenance of neurons and neuronal circuits. Apart from reports on reactive gliosis after auditory/vestibular injuries, few authors have focused their attention on the astroglial cytoarchytecture of the cochlear nuclei (CN). In this qualitative immunohistochemical study, we analyse the distribution of the astrocytic markers glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), and S-100 protein (S-100) in the adult CN of twelve young adult male rats.

GFAP-immunoreactive (GFAP-ir) astrocytes are abundant and heterogeneously distributed throughout the subdivisions of the CN, with preference in the granule cell domain areas. Cells immunostained for GS and S-100 are also present within the CN, but their patterns of distribution do not seem to fully resemble those seen for the GFAP-ir glial cells.

These results suggest a relationship between the distribution of astrocytes and the areas of glutamatergic and gabaergic inputs to the CN, suggesting that more than one morphological and functional type of astrocyte is present in the first central step of the auditory pathway.

Key words: Dorsal cochlear nucleus – Ventral cochlear nucleus – Granule cell domain – Glial fibrillary acidic protein – Glutamine synthetase – S-100 protein – Glutamate – GABA

INTRODUCTION

The cochlear nuclei (CN) are the first central step in the ascending auditory pathway. The neuronal cytoarchitecture of this nuclear complex has mainly been described in mammals (Ramón y Cajal 1909; Lorente de Nó, 1933; Harrison and Irving, 1965, 1966; Osen, 1969; Hackney et al., 1990). The CN include several main divisions; the ventral cochlear nucleus (VCN) –subdivided into an anterior ventral cochlear nucleus (AVCN), and a posterior one (PVCN)–, the dorsal cochlear nucleus (DCN), and the granule cell domain (GCD) or small cell shell (Ramón y Cajal, 1909; Lorente de Nó, 1933; Mugnaini et al., 1980a, b; Huston and Morest, 1996). The AVCN and PVCN are not homogeneous cytoarchitectonic divisions (Harrison and Irving, 1965, 1966; Osen, 1969; Hackney et al., 1990), and in most mammalian species studied the DCN is composed of three layers: 1 or molecular; 2 or fusiform (also known as pyramidal), and 3 or

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deep polymorphic region (Osen, 1969; Berrebi and Mugnaini, 1991). Finally the GCD comprises seven areas constituted by several types of small cells (Mugnaini et al., 1980a, b; Floris et al., 1994; Weedman et al., 1996).

Among the factors that depend on the functions of the central nervous system (CNS), the interactions between neurons and astrocytes play a key role. Thus, the supply of precursors for the synthesis of neurotransmitters (Hertz et al., 1999) or energy substrates for metabolism (Tsacopoulos and Magistretti, 1996; Magistretti and Pellerin, 1999; Magistretti, 2006), glutamate and GABA reuptake from the synaptic cleft (Minelli et al., 1995; Rothstein et al., 1996), protection against cytotoxicity by ammonium (Suárez et al., 2002), and the induction of blood-brain barrier properties to the capillaries of the CNS (Pekny et al., 1998) are all functions played by macroglial cells. In recent years, new research data have suggested that the functional involvements of the neuron/astrocyte interaction go beyond homeostasis and even reach the influence of the glia on synaptic transmission (Araque and et al., 1999; Halassa et al., 2007; Parri et al., 2007). Surprisingly, the non-neuronal cells of the mammalian auditory pathway did not attract the attention of researchers until the 90's of the last century. Studies on the presence of astrocytes in the CN have been reported for adult rats (Valderrama-Canales et al., 1993), also in the lateral superior olivary nucleus (Hafidi et al., 1994). The behaviour of glial cells after trauma, injury or aging has also been investigated in the auditory nuclei in several mammalian species (Jalenques et al., 1995, 1997; Insausti et al., 1999).

Thus, taking into account the relevant roles of astrocytes and the low number of studies related to the glia of the CN, here we focus on the qualitative distribution of astrocytes within the adult rat CN. Astrocytes were identified immunohistochemically by means of three antibodies directed against proteins located in astrocytes. Thus, a monoclonal antibody against GFAP, widely used as a specific astrocyte marker (Eng and Lee, 1995); a monoclonal antibody against glutamine synthetase, prominently localized in astrocytes (Norenberg and Martinez-Hernandez, 1979; Derouiche and Frotscher, 1991), and a polyclonal antibody against S-100, expressed mainly by astrocytes in the CNS (Reymond et al., 1996; Ogata and Kosaka, 2002) were used.

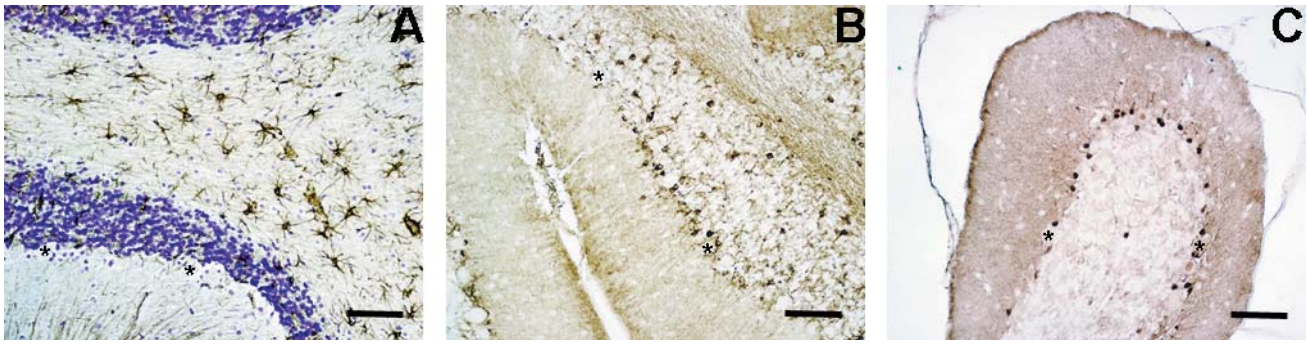
Material and Methods

Tissue preparation

Twelve young adult (3 months, 200-225g b/w) Long-Evans rats were used in this study. It is well known that the use of aldehyde-based fixatives affects the ability of the anti-GFAP antibodies to recognize their corresponding epitopes (Eng and DeArmond, 1981; Eng and Lee, 1995). Accordingly, in order to compare and discard artifactual immunohistochemical results owing to the fixation procedure, we used two different fixatives and performed two different methods of tissue processing for GFAP immunohistochemistry. GFAP antibodies of the clone GA5 afford better results if the tissue has been fixed with alcohol (Altmannsberger et al., 1982; Valderrama-Canales et al., 1993; Hafidi et al., 1994), but it has also been shown that some paraformaldehyde-based fixatives yield excellent results (Müller, 1992). Therefore, under proper aseptic conditions deeply anesthetized animals (pentobarbital, 60 mg/Kg, i.p.) were perfused transcardially with saline followed by either Zamboni s fixative (500 ml; 15% saturated aqueous picric acid, 4% paraformaldehyde solution in 0.1 M phosphate buffer saline –PBS–) or 2% acetic acid in ethanol (500 ml; Valderrama-Canales et al., 1993; Valderrama-Canales and Gil-Loyzaga, 2004). The fixatives for GS and S-100 immunohistochemistry were chosen according to previously published data on the nuclei of the auditory pathway: 2% acetic acid in ethanol for GS, and 4% paraformaldehyde in PBS for S-100 (Hafidi et al., 1994).

Brainstems were immediately dissected out and post-fixed overnight in the same perfusion solutions (2 hours for paraformaldehyde-fixed samples). Whereas Zamboni-fixed brainstems were either parasagittally or coronally sectioned at 50 µm in a vibratome, and the sections collected were carefully washed in cold PBS until the picric acid had been completely removed, the ethanol- and paraformaldehyde-fixed brainstems were included in paraffin. Blocks were sectioned at 10 µm, also in the coronal or sagittal planes, and the sections were mounted on slides covered with poly-L-lysine. For the immunohistochemical procedures, sections were deparaffinized and rehydrated.

Fig. 1. Positive controls for the three antibodies developed in the cerebellum. **A**: GFAP (alcohol-fixed tissue, counterstained with cresyl violet), **B**: GS (alcohol-fixed tissue), **C**: S-100 (formaldehyde-fixed tissue). Asterisks mark the position of Purkinje cells. Scale bar: 50 µm.



Antibodies and immunohistochemistry

The following primary antibodies were used: monoclonal anti-GFAP (Clone GA5; Boehringer Mannheim, Germany), monoclonal anti-GS (Chemicon, USA), and polyclonal anti-S-100 (Dako, Denmark). Unless otherwise stated, all immunohistochemical incubations were performed at room temperature. At the beginning of the protocol, endogenous peroxidase was blocked in all the sections with 0.3% H_2O_2 in PBS (20', gently agitation), after which the sections were rinsed with PBS.

Free-floating vibratome sections were pre-incubated with 30% normal horse serum (NHS) and 0.5% Triton X-100 (TX-100) in PBS for 30', and then incubated with 1:4 anti-GFAP in PBS containing 10% NHS, 5% sucrose, and 0.5% TX-100. After 3 days of incubation (4ºC), sections were thoroughly rinsed in PBS and further incubated with a horse biotinylated anti-mouse IgG (1:200; Vector, USA) in PBS (24 hours, 4ºC). Finally, the immunoreaction was visualized following the ABC-peroxidase technique (Vectastain, Vector, USA) and the sections were mounted and coverslipped.

The rehydrated deparaffinized sections were pre-incubated (30') with either 30% NHS (anti-GFAP and anti-GS antibodies) or 50% normal goat serum (NGS; anti-S-100 antibody) in PBS plus 0.1% TX-100. Incubations (24 hours, 4ºC) were performed with each selected antibody: anti-GFAP (1:4 in PBS plus 30% NHS and 0.1% TX-100), anti-GS (1:500 PBS plus 30% NHS and 0.1% TX-100), and anti-S100 (1:1000 in PBS plus 50% NGS and 0.1% TX-100). After a rinse in PBS, sections were further incubated with their corresponding biotinylated secondary antibodies (GFAP and GS: monoclonal horse anti-mouse IgG; S-100: goat anti-rabbit IgG; all from Vectastain, Vector, USA) 1:200 in PBS (24 hours, 4ºC). Finally, sections were processed with the ABC-peroxidase system (Vectastain, Vector, USA) as described for the vibratome sections. Some sections were lightly counterstained with cresyl violet.

Negative controls were performed by changing the primary antibody for the corresponding normal serum during the first incubation. No labelling was observed in any control section (not shown). Positive controls were carried out on cerebellum sections (Figs. 1A, 1B, 1C).

Results

Glial fibrillary acidic protein immunoreactivity

GFAP-immunoreactive (GFAP-ir) cells were observed throughout the CN (Fig. 2). In sections counterstained with cresyl violet, the cells had pale, spherical or ovoid nuclei, always devoid of immunoreaction, with some GFAP-ir processes emerging from their bodies (fig. 3). These cells were therefore considered to be astrocytes expressing GFAP (GFAP-astrocytes). There were no differences in the morphology and features of the GFAP-ir-astrocytes depending on the fixative employed. The neurons were consistently devoid of labelling. The *glia limitans* exhibited intense immunoreactivity, and the capillaries were enclosed by GFAP-ir end feet, some of them clearly belonging to processes coming from the GFAP-astrocytes (Fig. 3).

Anterior ventral cochlear nucleus

The general view of the AVCN revealed that its rostral region had the densest population of GFAP-astrocytes, whereas the rest of the nucleus had a low number of GFAP-astrocytes (Fig. 4A). At the rostral tip of the nucleus, where the spherical bushy cells were the

Fig. 2. View of the CN in a parasagittal section immunostained for GFAP. Note the uneven distribution of the GFAP immunoreaction. AVCN: anterior ventral cochlear nucleus, PVCN: posterior ventral cochlear nucleus, DCN: dorsal cochlear nucleus, VIII: root of the cochlear nerve. Alcohol-fixed tissue. Scale bar: 500 µm.

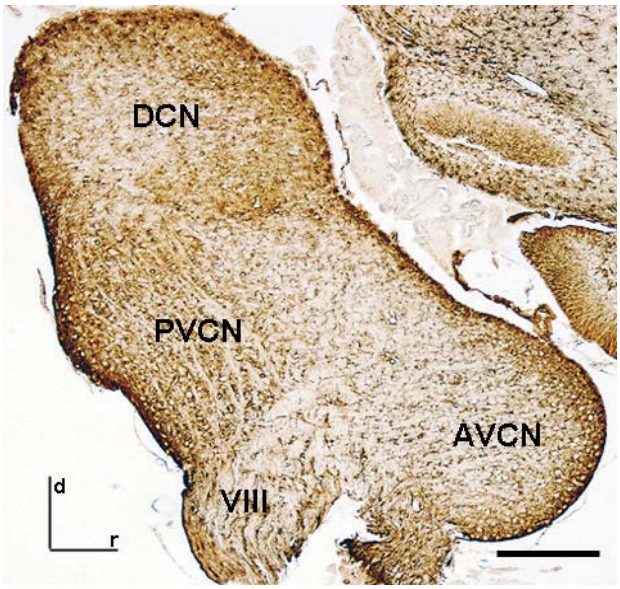
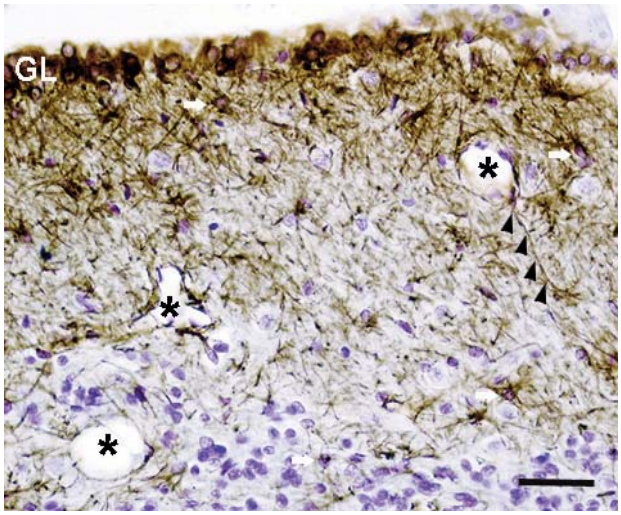


Fig. 3. Detail of a section of the CN immunostained for GFAP and counterstained with cresyl violet (alcohol-fixed tissue). GFAP-ir astrocytes, more densely grouped in the glia limitans (GL), can be seen within the tissue, sometimes with the nucleus well recognized because is devoid of GFAP-ir (white arrows). Asterisks are in capillaries, and one astrocyte process can be followed from the body of the astrocyte to the capillary (black arrowheads). Scale bar: 50 µm.



main neurons, the conspicuous immunoreaction was due to the presence of strong GFAP-ir astrocytes showing thick processes, occasionally wrapping the neuronal perikarya of putative spherical bushy cells (Fig. 4B). In the caudal region of the AVCN, which contains globular bushy and multipolar cells, the density of GFAP-astrocytes seemed to be lower than at the rostral tip of the nucleus (Fig. 4B), the astroglial cells showing apparently longer and thinner GFAP-ir processes. In the cochlear nerve root, the GFAP-astrocytes and their processes seemed to be packed among the branches of the cochlear nerve bifurcation (Fig. 5A), and some presumed cochlear nerve root neurons (Merchán et al., 1988) were covered by GFAP-astrocytes and their processes (Fig. 5B).

Posterior ventral cochlear nucleus

As in the AVCN, within the PVCN the immunoreaction was unevenly distributed but the GFAP-astrocytes appeared to be more abundant than in the AVCN and hence, the immunoreaction seemed to be more intense in the posterior area of the PVCN than in the anterior (Fig. 6A). In cresyl violet-counterstained sections some putative octopus cells were identified (Fig. 6B), together with globular bushy cells (Fig. 6C), and multipolar cells, frequently enfolded by some GFAP-astrocyte and its processes, whereas the descending branches of the cochlear nerve were easily recognized and were seen running in parallel bundles oriented dorsally to reach the DCN (Fig. 6A).

Dorsal cochlear nucleus

As in the VCN, the distribution of GFAP-ir-astrocytes was not homogeneous in the DCN (Fig. 7A). Strong GFAP-ir was observed in the molecular and fusiform (or 1 and 2) layers, and in the lamina of granule cells lying over the VCN but, in contrast, the deep region of the DCN gave the impression of a lower density of GFAP-ir astrocytes (Fig. 7B). In some sections, it was possible to observe the descending branches of the primary auditory afferents just entering the DCN, interrupting the dense GFAP-ir sheet corresponding to the layer of granule cells placed between the DCN and the VCN (Fig. 7C).

Granule cell domain (GCD)

The GCD, together with the molecular and fusiform layers of the DCN, appeared as the area with the highest density of GFAP-astrocytes, thus showing the most intense immunoreaction within the CN (Figs. 4A, 7A, 8A). Of the seven areas of the GCD, the lamina (Figs. 7A, 8A) and the superficial layer (Figs. 4A, 8B) always appeared sharply defined in all the sections where they were present, owing to their dense network of GFAP-astrocytes and processes.

Fig. 4. Parasagittal section of the AVCN immunostained for GFAP and counterstained with cresyl violet (alcohol-fixed tissue). **A**: The core of the nucleus has a lower number of GFAP-ir astrocytes than the tip. Scale bar: 50 µm. **B**: Detail of the rostral part of the nucleus showing the GFAP-ir astrocytes. Scale bar: 50 µm.

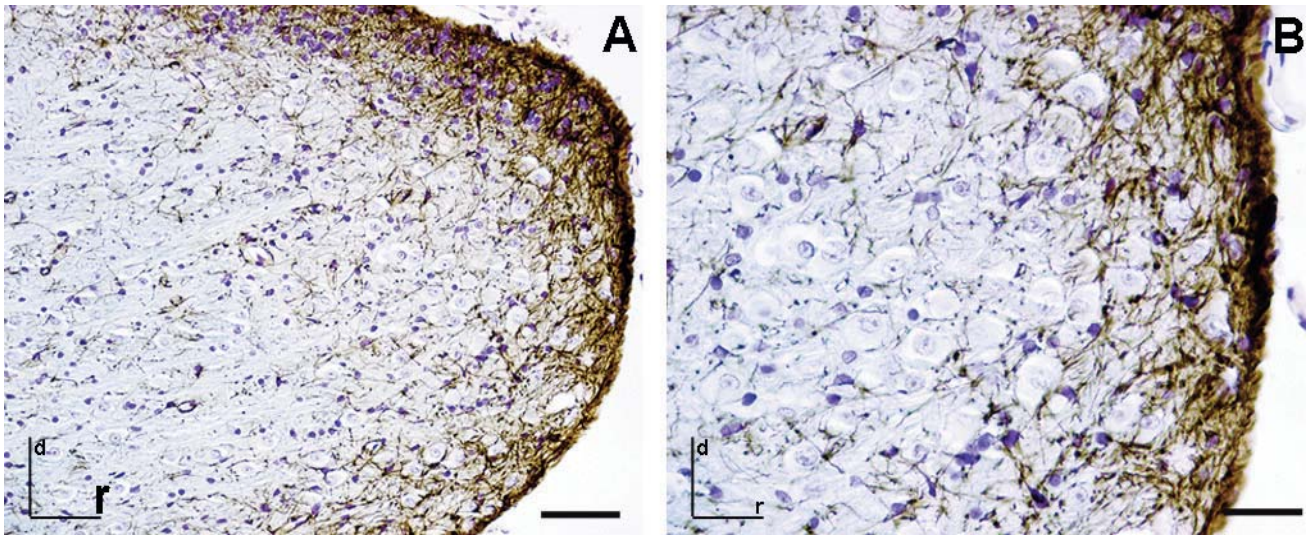


Fig. 5. Parasagittal section of the cochlear nerve root immunostained for GFAP and counterstained with cresyl violet (alcohol-fixed tissue). **A**: The bundles of primary afferents are delineated in a parallel array and GFAP-ir astrocytes can be seen among the bundles. Scale bar: 50 µm. **B**: A cochlear nerve root neuron surrounded by GFAP-ir astrocytic bodies and processes. Scale bar: 50 µm.

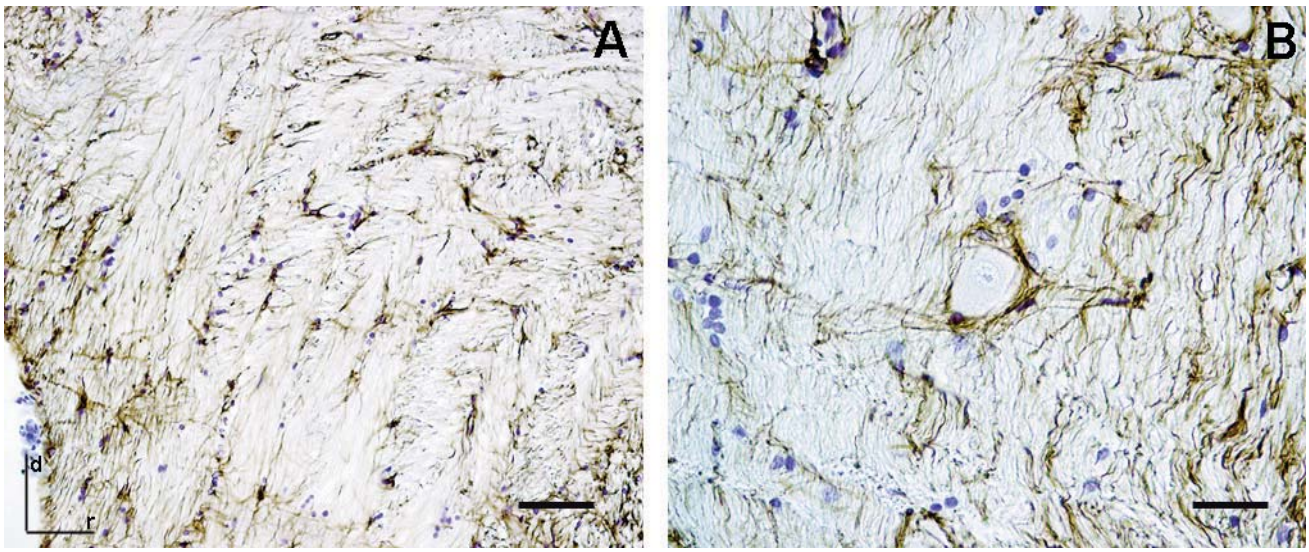


Fig. 6. General view and details of a parasagittal section of the PVCN immunostained for GFAP and counterstained with cresyl violet (alcohol-fixed tissue). **A**: The immunoreaction shows the presence of neuronal bodies and the descending branches of the cochlear nerve running to the DCN. Scale bar: 50 µm. **B**: A putative octopus cell body delineated by GFAP-ir astrocytes. Scale bar: 20 µm. **C**: This presumed globular bushy cell profile is also surrounded by GFAP-ir astrocytes. Scale bar: 20 µm.

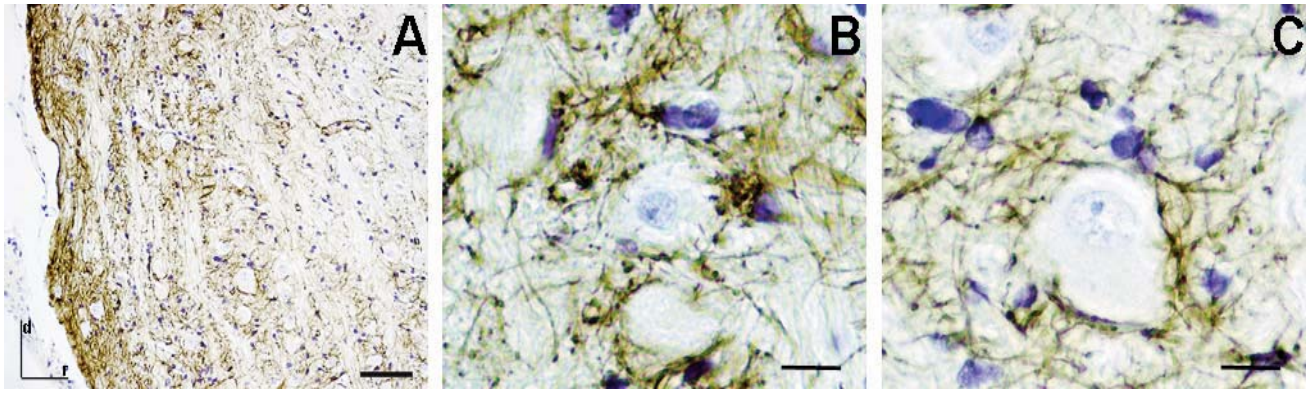


Fig. 7. General view and detail of a parasagittal section of the DCN immunostained for GFAP and counterstained with cresyl violet (alcohol-fixed tissue). **A**: The boundaries of the DCN are visible due to the dense population of GFAP-astrocytes, whereas the deep nucleus has a loose GFAP-ir astrocyte population. Scale bar: 100 µm. **B**: Detail showing GFAP-astrocytes of the layers 1 and 2, and some scattered GFAP-astrocyte in the deep nucleus. Scale bar: 50 µm. **C**: Scale bar: 50 µm.

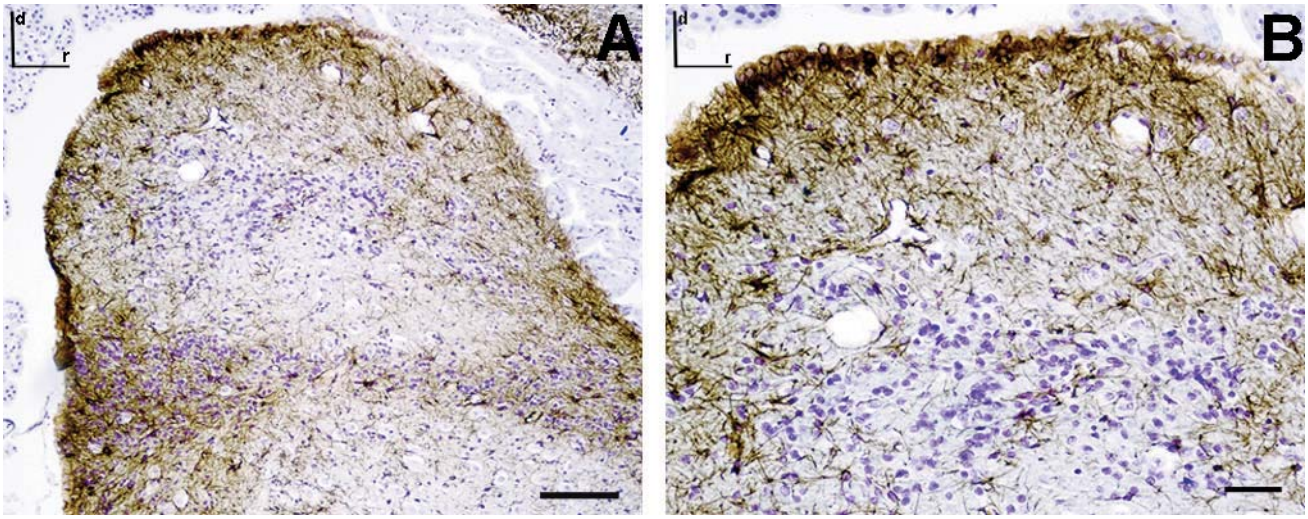


Fig. 8. **A**: General view and detail of a parasagittal section of the CN immunostained for GFAP and counterstained with cresyl violet (alcohol-fixed tissue) showing the lamina and the superficial layer of the GCD (both indicated by black arrowheads) as intense GFAP-ir areas. Scale bar: 500 µm. **B**: Imaging showing the GFAP-ir astrocytes of the superficial layer. Scale bar: 20 µm.

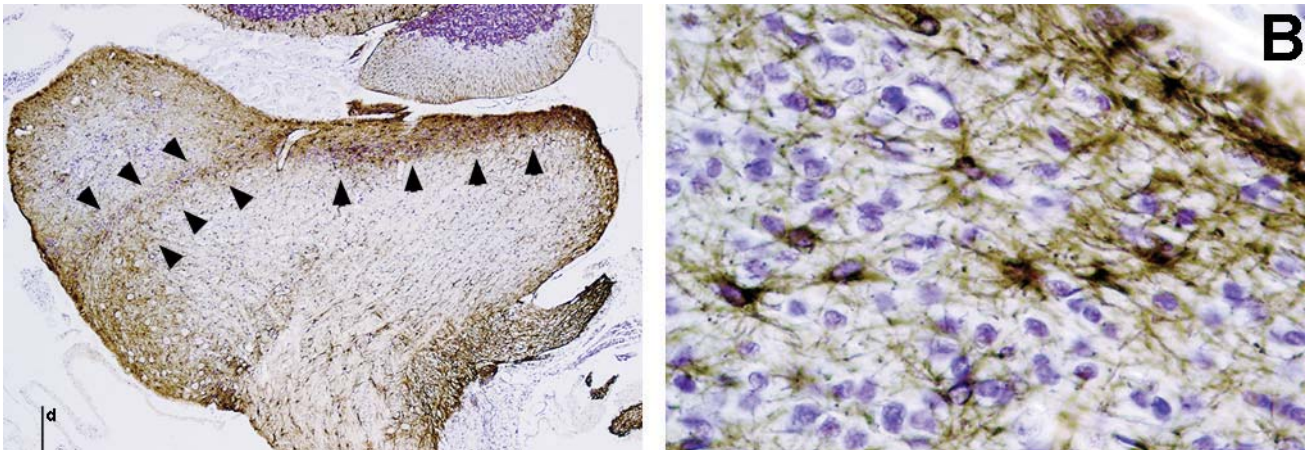
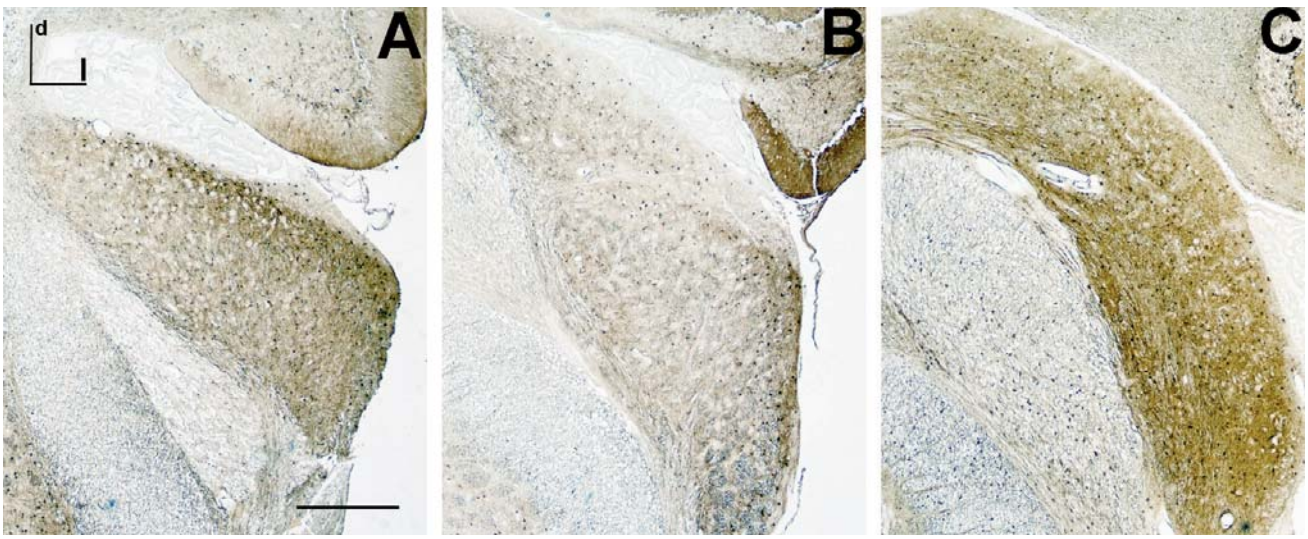


Fig. 9. View of the CN in a sequence of coronal sections immunostained for GS. GS-ir astrocytes appear as dark dots. Scale bar: 300 µm.



Glutamine synthetase immunoreactivity

GS-immunoreactive (GS-ir) cells were present in the VCN, DCN, and the GCD (Fig. 9A, B, C). GS-ir was confined to the cytoplasm of small non-neuronal cells, these being considered astrocytes expressing GS (GS-astrocytes). Occasionally, the initial segment of the processes was also GS-ir and, in addition, it was possible to distinguish fine GS-ir puncta distributed in all the divisions of the CN, sometimes accumulating and forming GS-ir patches (Fig. 10A, B). Some of the GS-astrocytes were observed adjacent to neuronal bodies, and the presence of couples or triplets of aggregated GS-astrocytes was observed sporadically (Fig. 10A, B).

S-100 immunoreactivity

As in the case of GFAP and GS, S-100 immunoreactive (S100-ir) cells were localised in all the divisions of the CN (Fig. 11A, B, C). In these cells, the immunoreaction, with a granulated aspect, was identified in the cytoplasm of the perikarya but also included the nucleoplasm, in agreement with the intranuclear distribution of S-100 (Donato, 2001). These S100-ir cells were analoguous in size and morphology to the GFAP- and GS-astrocytes, and were therefore considered to be S100-ir astrocytes (S100-astrocytes). Most S100-ir-astrocytes were located bordering neuronal somata, and there were frequently S100-ir puncta close to neuronal bodies (Fig. 12 A, B). Some S100-astrocytes could be

Fig. 10. Detail of the GS-ir astrocytes. The GS-ir appears as a cytoplasmic rim surrounding the nucleus always devoid of immunostaining. **A**: The arrows point to puncta delineating a neuronal profile. A couple of GS-ir astrocytes can be seen. Scale bar: 10 µm. **B**: Three GS-ir astrocytes are in apposition, forming a "triplet". Scale bar: 10 µm.

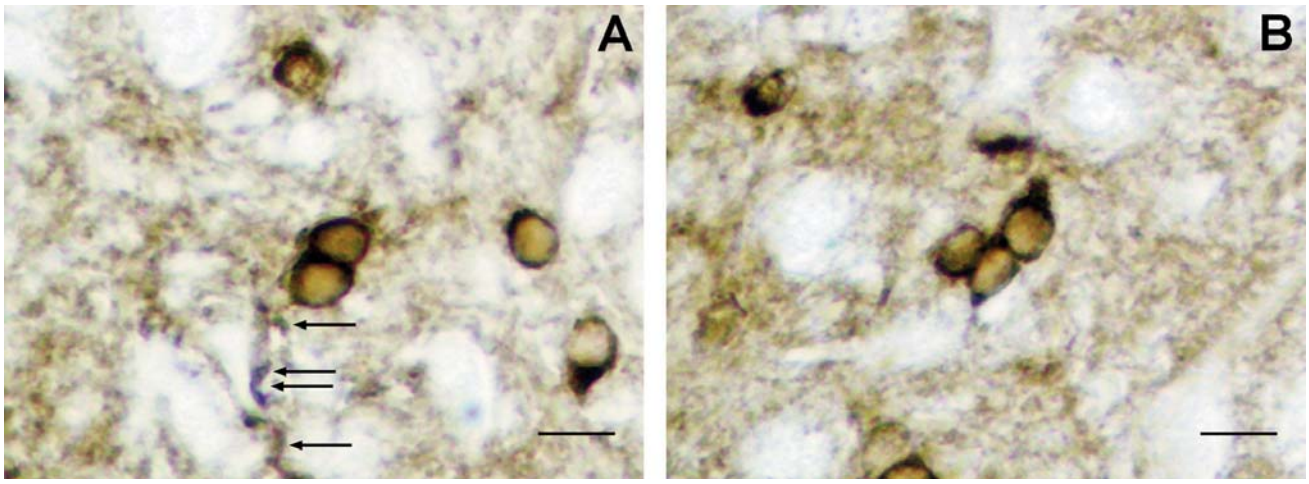


Fig. 11. View of the CN in a sequence of coronal sections immunostained for S-100. S-100-ir astrocytes appear as brown dots. Scale bar: 300 µm.

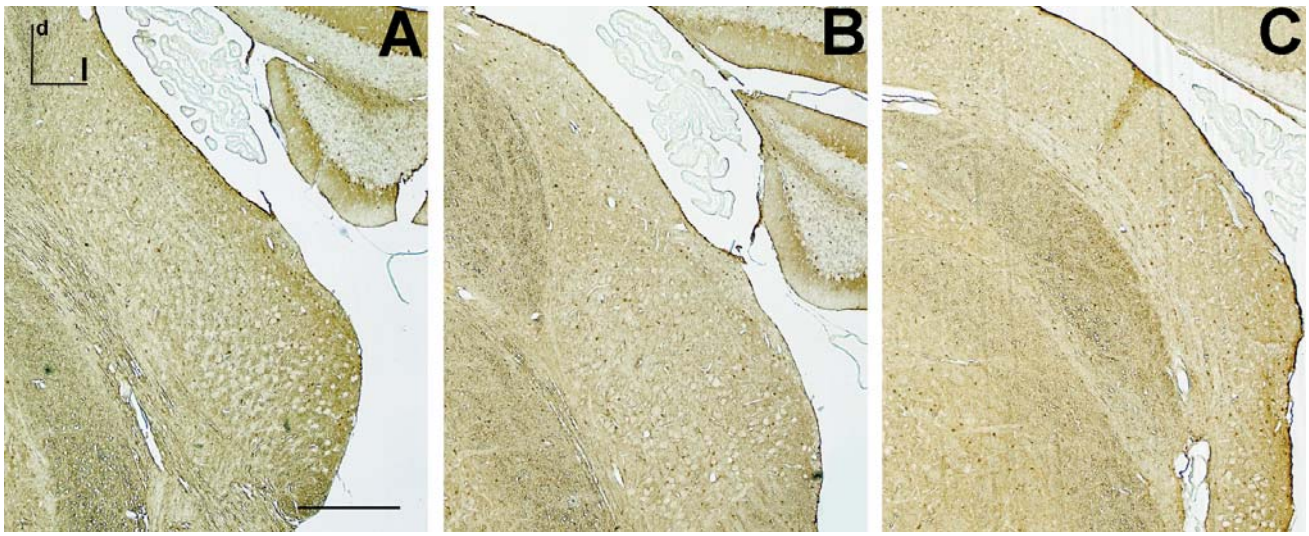
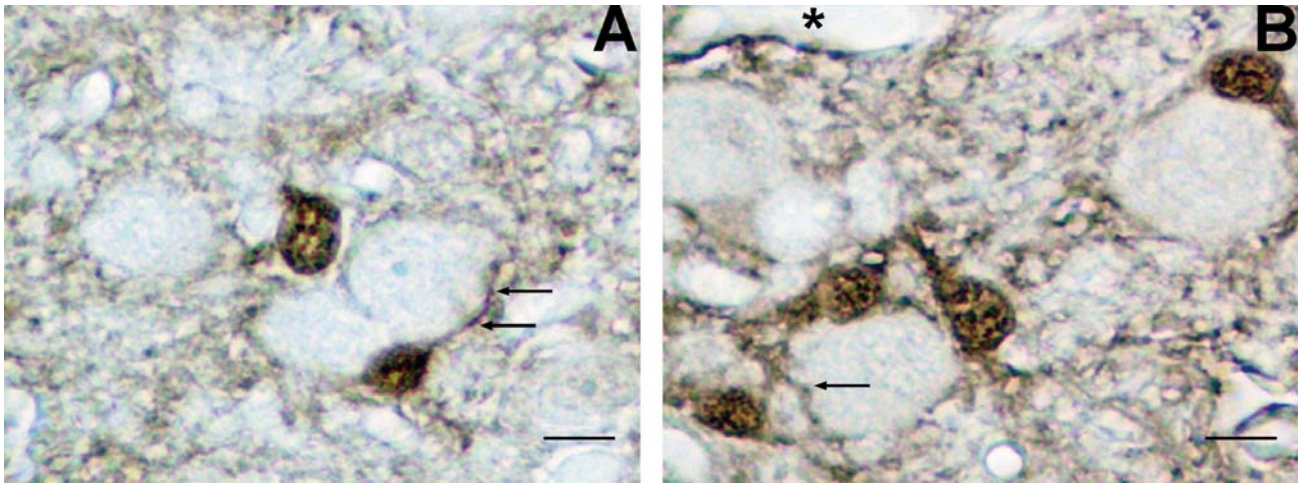


Fig. 12. Detail of S-100-ir astrocytes. The S-100-ir appears as a granulated product filling the cytoplasm and the nucleoplasm. All the S-100-ir astrocytes have a perineuronal location. The arrows point to puncta delineating neuronal profiles. No couples or triplets of S-100-ir astrocytes can be seen. The asterisk in B is in a capillar. Scale bar: 10 µm.



observed intermingled among bundles of fibres or adjacent to blood vessels, and these vessels often displayed a thin rim of S-100-IR lining the outer face of the vascular endothelium (Fig. 12 B).

Discussion

To identify the astrocytes of the CN, three different antibodies directed against astrocytic proteins were used, and all afforded a specific immunoreaction. Immunoreactive cells were located in all the divisions of the CN, but with an uneven distribution among the subnuclei as regards both the density of the cells and the pattern shown by the cells immunoreactive to each antibody.

Identification of astrocytes

Intermediate filament GFAP is expressed only by astrocytes within the CNS (Bignami et al., 1972; Ludwin et al., 1976; Dahl et al., 1985; Eng and Lee, 1995). The monoclonal anti-GFAP used in this study has been employed previously (Müller, 1992; Valderrama-Canales et al., 1993; Valderrama-Canales and Gil-Loyzaga, 2004), affording immunostained cells with the morphological characteristics of astrocytes as described on the basis of Golgi-impregnated material (Ramón y Cajal, 1913; Privat et al., 1995).

The GS has been identified in astrocytes elsewhere (Norenberg, 1979; Norenberg and Martinez-Hernandez, 1979; Derouiche and Frotscher, 1991; Derouiche and Rauen, 1995; Derouiche et al., 1996). The astrocytes identified by the monoclonal anti-GS used here are very similar to the astrocytes recognized with other anti-GS antibodies, including the striking presence of couples or triplets of cells and, consistently, nuclei free of immunoreaction (Norenberg, 1979; Hafidi et al., 1994). The failure in the staining of the astrocytic processes, in our tissue sections as well as in those reported by the above authors may be due to technical differences in the immunohistochemical protocol (Derouiche and Frotscher, 1991).

The term S-100 refers to a family of calcium-binding proteins (Donato, 2001) that, within the CNS, are expressed preferentially by astrocytes (Ludwin et al., 1976; Reymond et al., 1996; Ogata and Kosaka, 2002). The anti-S-100 polyclonal antibody used in our study exclusively recognizes astrocytes (Müller, 1992; Tanaka et al., 1992) and the pattern of the S100-ir is maintained, regardless of the fixation method (Dyck et al., 1993). Consistent with this, the S100-astrocytes identified in our experiments resembled those found in similar studies (Hafidi et al., 1994), and maintained the main immunoreaction features, such as the nuclear staining and the morphology of the body, when compared with reports in which the nervous tissue was fixed with alcohol-based fixatives (Müller, 1992).

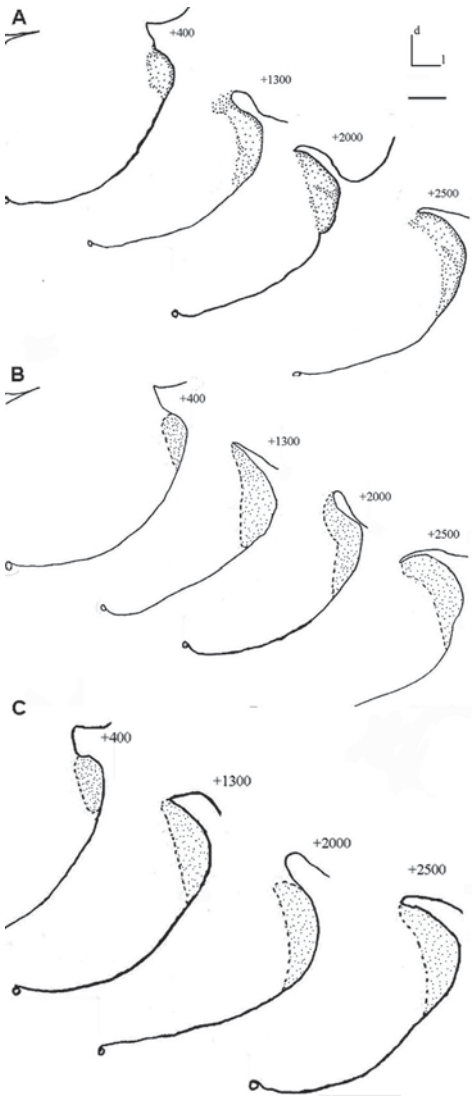
Distribution of the astrocytes: is there more than one phenotypic population of astrocytes within the CN?

Our results point to an uneven distribution of GFAP-astrocytes within the adult rat CN. The population of GFAP-astrocytes seemed to

be denser in the GCD, the molecular and fusiform layers of the DCN, and in the slim subpial, or subependymal, sheet forming the *glia limitans*. In contrast, the deep nucleus of the DCN and the AVCN appeared to have a lower population of GFAP-ir astrocytes. These results reflect a heterogeneous distribution of the GFAP-ir astrocytes within the CN that could be due to the diverse distribution of the GFAP-ir astrocytes among the divisions of the CN, to the presence of diverse subpopulations of astrocytes displaying differential GFAP expression, or both. Previous immunohistochemical studies performed on the rat CN have described the GCD together with the superficial layers of the DCN as those most densely occupied by GFAP-ir astrocytes (Valderrama-Canales et al., 1993). Later, the authors of quantitative studies addressing the aging process of the rat VCN concluded that the densest population of GFAP-ir astrocytes lies on the GCD (Jalenques et al., 1995, 1997). In the CNS it has been demonstrated that GFAP immunostaining has a heterogeneous pattern (Bignami et al., 1972; Ludwin et al., 1976; Hajós and Kálmán, 1989; Kálmán and Hajós, 1989; Zilles et al., 1991; Bailey and Shipley, 1993), with strong evidence pointing to the notion that the structures developed in the embryonic pial and ventricular surfaces have the densest accretion of GFAP-ir astrocytes (Ludwin et al., 1976; Zilles et al., 1991), also in the CN (Burette et al., 1998). This type of development occurs for the CN (Taber, 1967), and hence can explain the heavy subpial and subependymal GFAP-ir. Electron microscopy studies on the neuronal types of the DCN have reported the presence of astrocytes in layers 1 and 2 (Mugnaini et al., 1980b), with measurements that strongly support the fact of a dense astrocytic population in these layers (Wouterlood and Mugnaini, 1984). Regarding the presence of different populations of astrocytes according to their phenotype, it is known that the GFAP-ir astrocytes represent only one subset of all astrocytes (Walz and Lang, 1998), whereas others do not express GFAP or do so in lower amounts unamenable to recognition by immunohistochemistry (Eng and Lee, 1995). Consequently, it is possible that there are astrocytic populations in the CN that do not express GFAP. In other auditory nuclei the asymmetrical distribution between GFAP-ir astrocytes has been well documented (Hafidi et al., 1994).

The distribution of the GS- and S100-astrocytes in the CN does not fit the pattern for the GFAP-ir astrocytes (Fig. 13). Discordances in the arrangement of GS- and GFAP-astrocytes have been reported previously throughout the CNS (Hallermayer and Hamprecht, 1984; Patel et al., 1985; Didier et al., 1986; Tanaka et al., 1992; Walz and Lang, 1998), including auditory nuclei (Hafidi et al., 1994; Burette el al., 1998). The striking presence of pairs or triplets of GS-astrocytes within the CN has also been noticed in the lateral superior olive (Hafidi et al., 1994) as well as in other CNS areas (Norenberg, 1979). Nothing has been suggested about the func-

Fig. 13. Rostrocaudal sequences along the CN to illustrate the distribution of the GFAP-ir astrocytes (**A**), the GS-ir astrocytes (**B**), and the S-100-ir astrocytes (**C**); all populations are represented by black dots. Scale bar: 500 µm. d: dorsal, l: lateral. **A**: The distribution of the GFAP-ir astrocytes shows the formation of some boundaries rich in GFAP-ir astrocytes that separate neighbouring areas. **B** and **C**: GS-ir- and S-100-ir astrocytes seem to be more "randomly" distributed and there are no defined limits among the several areas of the CN.



tional role of these cell aggregates, but they have never been described either for the GFAP-ir astrocytes or for the S100-ir astrocytes. The distribution of S100-astrocytes within the CN cannot be attributed to a loss of antigenicity derived from the fixation method since fixatives do not affect the patterns of immunostaining (Dyck et al., 1993). Nevertheless, as for the GS-ir- and GFAP-ir astrocytes, different patterns of distribution between S100-ir- and GFAP-ir astrocytes have been described in several territories of the CNS (Ludwin et al., 1976; Didier et al., 1986; Tanaka et al., 1992; Gary and Chronwall, 1995), including the auditory nuclei (Hafidi et al., 1994; Burette et al., 1998). Thus, it is possible that several phenotypic populations coexist in the CN. Studies addressing astrocytes *in vitro* and *in vivo* (Hallermayer and Hamprecht, 1984; Didier et al., 1986; Aoki et al., 1987; Bailey and Shipley, 1993) have demonstrated considerable astrocytic diversity, and it has been proposed that GS would be expressed only by protoplasmic astrocytes (Didier et al., 1986).

Functional implications

Our results indicate that in the CN, as in other regions of the CNS, astrocytes may play a role in compartmentalization among several regions. The presence of glial boundaries within the CNS has been confirmed both in adults (Steindler and Cooper, 1986; Suzue et al., 1990; Bailey and Shipley, 1993) and during development (Steindler, 1993). It is reasonable to surmise that these boundaries would be related to the cytoarchitecture of the CN, since the laminated arrangement of the astrocytes matches the laminar cytoarchitecture of the olfactory bulb (Bailey and Shipley, 1993). At cellular scale, the intimate relationship of neurons and astrocytes is well known (Lafarga et al., 1984; Raisman, 1985; Kosaka and Hama, 1986; Steindler and Cooper, 1986; Valverde and López-Mascaraque, 1991). In the CN, the GCD is the subdivision with the highest density of astrocytes and it is in the GCD where descriptions have been made of synaptic nests, clusters of synaptic endings not individually contained by astrocytic processes but all of them grouped and surrounded by astrocytes (Huston and Morest, 1996, Hurd et al., 1999). Thus, it could be speculated that the special presence of astrocytes within the GCD would be correlated with synaptic nests.

The crucial importance of astrocytes in the reuptake and processing of glutamate has been confirmed (Rothstein et al., 1996; Hertz et al., 1999), and strong immunohistochemical evidence supports the idea that astrocytic processes containing GS ensheathed glutamatergic synapses (Derouiche and Frotscher, 1991; Derouiche and Rauen, 1995; Derouiche et al., 1996). The cochlear nerve provides glutamatergic input (Wenthold, 1985) to the bushy cells in the AVCN (Fekete et al., 1984), the octopus cells in the PVCN (Fekete et al., 1984), and the pyramidal cells in the DCN (Ryugo and May, 1993). The GCD also receives glutamatergic input from the cochlear nerve type II fibres (Hurd et al., 1999), the auditory cortex (Weedman and Ryugo, 1996), and non-auditory projections (Weedman et al., 1996; Wright and Ryugo, 1996). Moreover, the cochlear granule cells of the GCD organize a glutamatergic circuit in the molecular layer of the DCN (Osen et al., 1995). Subsequently, the presence of GS-astrocytes in the CN probably reflects the extensive glutamatergic transmission established in the neuronal circuits of the CN. In addition to these excitatory inputs, a number of intrinsic and extrinsic inhibitory systems mediated by GABA project within the CN (Mugnaini, 1985; Juiz et al., 1996; Moore et al., 1996). This GABAergic transmission could also contribute to the important presence of GS-astrocytes throughout the CN since glutamine is the major precursor of GABA synthesis and is provided to neurons by astrocytes (Battaglioli and Martin, 1991).

Among the various roles proposed for S-100 in the CNS, the most remarkable are inhibition of GFAP assembly (Bianchi et al., 1993), trophic properties for both neurons and glia (Winningham-Major et al., 1989; Selinfreund et al., 1991), and the stimulation of astrocytic and neuronal apoptosis mediated by nitric oxide (Hu and Van Eldik, 1996; Hu et al., 1997). Hence, within the CN S-100 could regulate GFAP assembly, contribute to maintaining and preserving the neuronal circuitry and, under some conditions, to regulating neuronal and glial apoptosis. These roles have also been proposed for S-100 in the lateral superior olive (Hafidi et al., 1994).

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