Biotin Is Endogenously Expressed in Select Regions of the Rat Central Nervous System

BRUCE E. MᴄKAY, MICHAEL L. MOLINEUX, AND RAY W. TURNER*

Neuroscience Research Group, University of Calgary, Calgary, Alberta T2N 4N1, Canada

ABSTRACT

The vitamin biotin is an endogenous molecule that acts as an important cofactor for several carboxylases in the citric acid cycle. Disorders of biotin metabolism produce neurological symptoms that range from ataxia to sensory loss, suggesting the presence of biotin in specific functional systems of the CNS. Although biotin has been described in some cells of nonmammalian nervous systems, the distribution of biotin in mammalian CNS is virtually unknown. We report the presence of biotin in select regions of rat CNS, as revealed with a monoclonal antibody directed against biotin and with avidin- and streptavidin-conjugated labels. Detectable levels of biotin were primarily found caudal to the diencephalon, with greatest expression in the cerebellar motor system and several brainstem auditory nuclei. Biotin was found as a somatic label in cerebellar Purkinje cells, in cell bodies and proximal dendrites of cerebellar deep nuclear neurons, and in red nuclear neurons. Biotin was detected in cells of the spiral ganglion, somata and proximal dendrites of cells in the cochlear nuclei, superior olivary nuclei, medial nucleus of the trapezoid body, and nucleus of the lateral lemniscus. Biotin was further found in pontine nuclei and fiber tracts, the substantia nigra pars reticulata, lateral mammillary nucleus, and a small number of hippocampal interneurons. Biotin was detected in glial cells of major tract systems throughout the brain but was most prominent in tracts of the hindbrain. Biotin is thus expressed in select regions of rat CNS with a distribution that correlates to the known clinical sequelae associated with biotin deficiencies. J. Comp. Neurol. 473:86–96, 2004.   © 2004 Wiley-Liss, Inc.

Indexing terms: cochlear nucleus; Purkinje cells; cerebellum; deep cerebellar nuclei; auditory nuclei; immunocytochemistry; avidin; streptavidin; biotin; MNTB

Biotin is a small vitamin molecule that acts as a covalently attached prosthetic group on at least four important carboxylases involved in oxidative metabolism, either through the citric acid cycle or via the synthesis or degradation of specific fatty acids and amino acids (Moss and Lane, 1971; Wood and Barden, 1977; Dakshinamurti and Chauhan, 1989). Biotin is processed through a cycle where it is first linked to carboxylases by the enzyme holocarboxylase synthetase and ultimately recycled following release by biotinidase (Thoma and Peterson, 1954; Wolf, 2001). The importance of this molecule to neuronal function is revealed when disruption of this cycle produces multiple carboxylase or biotinidase deficiencies that lead to serious sensory and motor deficits (Wolf and Feldman, 1982; Wolf et al., 1983, 1985).

High levels of biotin are present in kidney and liver cells (Bhagavan and Coursin, 1970; Wood and Warnke, 1981; Wang and Pevsner, 1999), and in select cells of nonmammalian nervous systems (Ma, 1994; Ziegler et al., 1995;

Eisner et al., 1996; Bhattacharjee et al., 1997; Johnson et al., 2000; Berkowitz, 2002). Several lines of evidence suggest that biotin is also expressed within the mammalian CNS. First, disorders of biotin metabolism generate characteristic neurological symptoms, including sensorineural

hearing loss, ataxia, and tremor (Wolf and Feldman, 1982; Suchy et al., 1985; Wolf et al., 1983, 1985; Wolf, 2001). Second, active carrier-mediated transport mechanisms raise biotin in CSF to levels 50–250% greater than plasma, and maintain the activity of biotin-dependent enzymes in brain even during challenge with low dietary biotin (Bhagavan and Coursin, 1970; Chiang and Mistry, 1974; Sander et al., 1982; Spector and Mock, 1987, 1988; Lo et al., 1991). Third, biotinidase has specific activity in brain and has been localized to select cell types (Oizumi and Hayakawa, 1989, 1990; Heller et al., 2002). Despite these results, little is known of the distribution of biotin in rat CNS. Two reports indicated the expression of biotin in a limited number of oligodendrocytes and scattered neurons in the most rostral regions of rat forebrain (LeVine and Macklin, 1988; Wang and Pevsner, 1999). However, no data have been presented on the potential for biotin to be expressed at detectable levels in any structure caudal to the hippocampus.

The present study reports the expression of biotin in specific nuclei of rat CNS, revealed through use of an antibiotin antibody or through direct labeling of biotin with avidin/streptavidin conjugates. Biotin was found primarily caudal to the diencephalon, with little or no neuronal label in forebrain regions. The highest level of biotin was in motor nuclei related to cerebellar function and in brainstem nuclei of the auditory system, consistent with clinical symptoms that follow disruption of biotin metabolism. Endogenous biotin has further implications for the accurate detection of antigens when applying immunocytochemical procedures that rely on avidin–biotin interactions.

MATERIALS AND METHODS
Animal care

Male Sprague-Dawley rats (4–6 weeks) were obtained from Charles River, Canada, and maintained on standard lab rations. All procedures were approved by the local Animal Care Committee and were in accordance with the guidelines established by the Canadian Council for Animal Care (CCAC). In total, over 30 animals were used in the course of completing three separate tests for biotin and their associated controls.

Histology

All chemicals were obtained from Sigma (St. Louis, MO) and immunochemicals from Vector Laboratories (Burlingame, CA) unless otherwise indicated. Rats were deeply anesthetized with an overdose of sodium pentobarbital and perfused intracardially with 250 ml of 0.1M phosphate buffer (PB, pH 7.4) followed by 100 ml of freshly prepared 4% paraformaldehyde (PARA, pH 7.4); both solutions were administered at room temperature. Brains were quickly removed, placed into 4% PARA at room temperature for 1 hour, and left overnight at 4°C. Free-floating 30–40 μm sections were cut by vibratome in ice-cold PB 24 hours later. Slices were transferred into either PB or 2% PARA and stored at 4°C until use (24–48 hours following slicing for PB-stored tissue, 1 week for 2% PARA-stored tissue). Working solutions consisted of 3% normal donkey or horse serum, depending on the species in which the secondary antibody was raised (Jackson ImmunoResearch, West Grove, PA), 0.1% Triton X-100, bal-ance PB. Gentle agitation to ensure thorough washing or exposure of tissue sections to antibodies or markers was applied throughout all experiments.

Tissue sections stored in PB were washed 3 × 20 minutes in working solution and those in PARA 4 × 60 minutes in PB prior to working solution washings. Primary antibody (mouse antibiotin, Sigma, 1:500) was included in the working solution for 24 hours at 4°C. The tissue sections were washed 3 × 20 minutes in PB to remove any unbound primary antibody, and then 3 × 20 minutes in working solution. Sections were incubated for 24 hours at 4°C in working solution containing any of AlexaFluor 488-conjugated donkey antimouse IgG (1:1,000; Molecular Probes, Eugene, OR), Cy3-conjugated donkey antimouse IgG (1:1,000), or biotinylated horse antimouse IgG (1: 1,200). Sections were then washed 3 × 20 minutes in PB. For tissue exposed to either AlexaFluor 488- or Cy3-conjugated secondary antibodies, sections were washed and then immediately mounted on gel-coated slides and coverslipped with antifade medium (90% glycerol/PBS/ 0.1% p-phenylenediamine; pH 10), sealed with nail polish, and stored at –20°C. Tissue exposed to the biotinylated horse antimouse IgG was treated with streptavidin-Cy3 (1:1,500) for 4 hours at room temperature in the dark, then mounted and stored as described above. Alternatively, tissue exposed to the biotinylated horse antimouse IgG was treated with the Vector ABC Elite HRP kit. HRP was visualized by incubating sections for 10 minutes in 3,3'-diaminobenzidine (DAB, 0.7 mg/ml), 20 μl glucose oxidase, 40 μl ammonium chloride, and 160 μl βD+-Glucose in 20 ml PB. Sections were mounted on poly-D-lysine gel-coated slides, dried overnight, processed through an ethanol and xylene series, and coverslipped in Entellan. Given the high isoelectric point (pI 10) of avidin, PB for reactions employing the Vector Elite kit included 0.5 M NaCl (PBS) to minimize nonspecific interactions (Guesdon et al., 1979).

Imaging

HRP-labeled sections were imaged on an Olympus BH-2 research microscope using a CCD camera (Photometrics Cool-Snap cf) and stored on a Pentium computer. Fluorescent labeling was imaged on an Olympus FV300 BX50 confocal microscope with FluoView software. Images were transferred to Adobe PhotoShop 6.0 and Adobe Illustrator 9.0 (San Jose, CA) for figure preparation, with image adjustments restricted to intensity levels or brightness/contrast. Identical camera settings and level-contrast adjustments were used for pairs of control and test photomicrographs during image processing. For all experiments comparing relative levels of biotin expression, adjacent sections from the same brain were used to complete any given figure. There was complete agreement between our classifications of cell types as positive or negative for biotin with that of an experienced histologist under single-blind conditions.

RESULTS
Biotin detection in rat CNS

Three methods were used to detect biotin and verify the accuracy of detection. The most conventional approach was to use a monoclonal antibody directed against biotin. Control reactions included preabsorbing the antibiotin an-

TABLE 1. Distribution of Biotin within Neurons of the Rat CNS[1]

Motor system	
Cerebellum	
Purkinje	+
granule	−
deep cerebellar	+
Golgi	−
molecular layer interneurons	−
Brain stem	
neurons of the pontine nuclei	+
red nucleus	+
Auditory system	
spiral ganglion	+
dorsal cochlear nucleus	+
ventral cochlear nucleus	+
superior olivary nucleus	+
medial nucleus of the trapezoid body	+
lateral lemniscus	+
inferior colliculus	−
medial geniculate nucleus	−
auditory cortex	−
Other rostral brain structures	
substantia nigra pars reticulata	+
lateral mammillary nucleus	+
hippocampal interneurons	+
glial cells of the optic tract	+

[1]Nuclei containing cells positive (+) or negative (−) for endogenous biotin detected using an antibiotin antibody, streptavidin-Cy3, or the avidin-biotin complex of the Vector Elite kit.

tibody for 20 minutes with an excess of free biotin (0.1% free biotin) or by omitting the primary antibody. A second approach was to apply streptavidin-Cy3 alone to directly label sites of endogenous biotin. The final method was to apply an avidin-biotin complex (ABC; Vector Elite) in the absence of any immunoreagents and use DAB to visualize the horseradish peroxidase (HRP) conjugate. All sections entered into the Vector Elite procedure were first incubated in 0.3% H_2O_2 to destroy endogenous peroxidase activity. Control reactions for avidin- or streptavidin-conjugated markers were completed using commercially available avidin- and streptavidin-biotin blocking kits.

Each of these techniques revealed biotin expression in specific nuclei, with complete overlap in colabel experiments using a mouse antibiotin antibody (visualized with donkey antimouse–AlexaFluor 488) and streptavidin-Cy3. All neuronal labeling was bilaterally symmetric and cell types identified as biotin-positive were repeatedly labeled in different experiments. Avidin and streptavidin conjugates could produce an additional diffuse and amorphic background label in some nuclei (considered in more detail below). Since this label was not confirmed with the antibiotin antibody it was not included in our description of biotin-labeled neuronal cell types.

Biotin distribution

Previous assessments of biotin distribution in rat CNS were restricted to tissue sections taken exclusively from rostral forebrain (LeVine and Macklin, 1988; Wang and Pevsner, 1999). We extended these studies by examining biotin expression in over 100 structures throughout the rat brain (described in Paxinos and Watson, 1986). As previously reported, only weak levels of biotin label were detected in scattered hippocampal interneurons or glial cells in forebrain. However, we found biotin in several hindbrain nuclei, and in key motor and sensory systems (Table 1).

Cerebellar motor system

Biotin label was restricted to the cell bodies of cerebellar Purkinje cells (Fig. 1A–C). At low magnification, all Pur-

kinje cells were labeled evenly around the 10 lobules visible in sagittal sections (Fig. 1D), with no evidence for parasagittal stripes in coronal sections. Granule cell bodies were negative, but were surrounded by a weak and diffuse label of undetermined origin (Fig. 1A–C). No specific label was detected in stellate, basket, or Golgi cells (data not shown).

Biotin was also present in all cerebellar nuclei. Enough cells were labeled that even at low magnification one could detect a loose matrix of cells within the confines of a nucleus (Fig. 1E). Higher magnification revealed biotin label in the cell bodies of both small and large diameter cerebellar nuclear neurons (Fig. 1F). No evidence was obtained for biotin labeling of the prominent Purkinje cell axon synaptic boutons that outline deep cerebellar neurons.

Biotin label was expressed in neurons of the red nucleus (Fig. 1G) but not in thalamic nuclei or other definable cerebellar projection sites. Finally, high-intensity label was detected in neurons of the pontine nuclei, suggesting that some nuclei giving rise to afferent mossy fibers are positive for biotin (Fig. 1H). The inferior olivary nucleus was biotin-negative.

Neurons in other motor centers did not express biotin, including motor cortex, basal ganglia, motor nuclei of thalamus, and spinal motoneurons. However, biotin was detected in many small fascicles projecting along the rostrocaudal axis of the brainstem, consistent with axons or oligodendrocytes associated with the corticospinal, corticobulbar, and corticopontine fiber tracts.

Auditory nuclei

Biotin was clearly expressed in cells of several brainstem auditory nuclei. Biotin was localized to neuronal cell bodies and processes in the spiral ganglion (Fig. 2A) and was widely expressed in larger cells of both the dorsal and ventral cochlear nuclei (Fig. 2B). The superior olivary nuclei exhibited a high-intensity label of neuronal cell bodies (Fig. 2C). The highest level of biotin expression in the entire brain was found in the medial nucleus of the trapezoid body (MNTB) (Fig. 2D). Biotin was also expressed in cells of the nucleus of the lateral lemniscus, with a clear visualization of columns of cells against the unlabeled background of axon projections through the nucleus (Fig. 2E). Little or no biotin was detected in the inferior colliculus, medial geniculate body, or auditory cortex (Table 1).

Biotin distribution in rostral brain regions

Biotin was clearly expressed in neurons of the substantia nigra pars reticulata (Fig. 3A) and the lateral mammillary nucleus (Fig. 3B). Within the hippocampus, only a few interneurons were weakly positive for biotin in any given tissue section (Fig. 3C). When found, these interneurons were juxtaposed among pyramidal cells of the CA fields and granule cells of the dentate gyrus. No biotin label could be detected in nuclei associated with the visual system, including lateral geniculate and visual cortex. Biotin is also known to be expressed by glial cells (LeVine and Macklin, 1988). We found glial-like labeling in several major tract systems, such as the optic tract, anterior commissure, and in the spinal trigeminal tract (Fig. 3D–F).
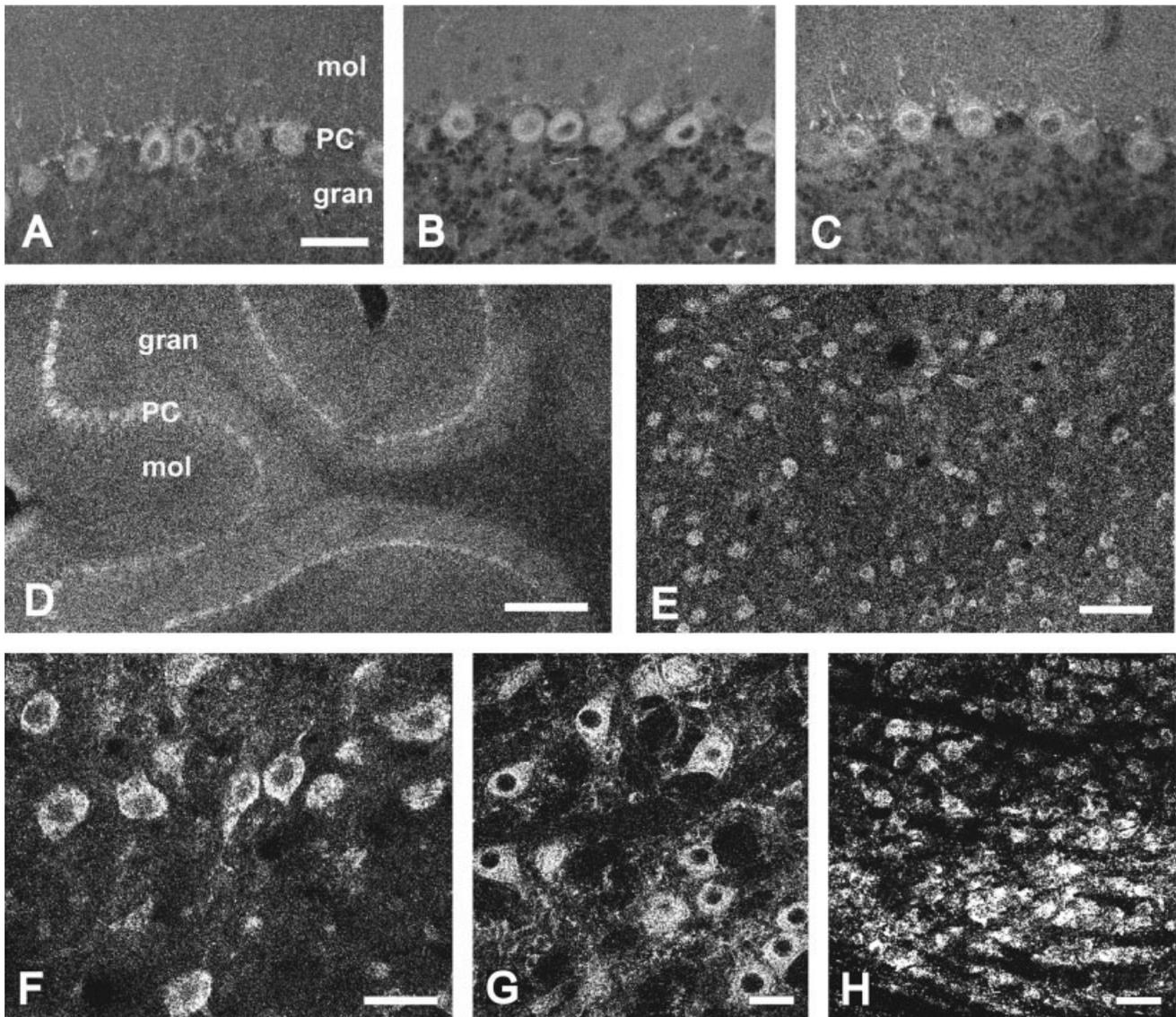
Fig. 1. Endogenous biotin expression in select nuclei of the cerebellar motor system. **A:** The combination of antibiotin antibody, a biotinylated secondary antibody, and streptavidin-Cy3 prominently label the somata of cerebellar Purkinje cells. **B,C:** Identical labeling was noted using a biotinylated secondary antibody and streptavidin-Cy3 (B) or streptavidin-Cy3 alone (C). **D:** Low magnification of sagittal cerebellar section indicating homogenous Purkinje cell somatic labeling around the lobules. **E:** Biotin-positive neurons within a deep cerebellar nucleus. **F:** At higher magnification both small and large deep cerebellar nuclear cell types are biotin-positive. **G:** Biotin is found in the somata of neurons within the red nucleus. **H:** Cells of the pontine nuclei are biotin-positive. Label in D and G was revealed by streptavidin-Cy3; E,F,H by antibiotin antibody and a Cy3- or Alexa-Fluor 488-conjugated secondary antibody. PC, Purkinje cell layer; mol, molecular layer; gran, granule cell layer. Scale bars = 40 μm in A (applies to A–C); 50 μm in F (applies to F–H); 100 μm in E; 200 μm in D.

Biotin expression and immunocytochemistry

Biotin is best known for its extensive use in the avidin–biotin complex (ABC) reaction that forms the basis for much of immunocytochemistry. Endogenous biotin thus has the potential to produce false-positive results when applying avidin or streptavidin conjugates. In fact, this is problematic in clinical assays of kidney and liver cells, which express high levels of endogenous biotin (Bratthauer, 1999). As a result, methods to block the binding of avidin to endogenous biotin were described shortly after the introduction of the ABC technique (Wood and Warnke,

1981). These include biotin blocking kits and the reported loss of biotin label after paraformaldehyde fixation (Hsu, 1990; Wang and Pevsner, 1999). It has also been reported that endogenous biotin is more readily identified in rat stomach and frog brain when the membrane-permeabilizing detergent Triton X-100 is incorporated into immunochemical procedures (Satoh et al., 1992; Eisner et al., 1996). We therefore used the above three protocols to test their effect on biotin label in the cerebellum and MNTB. Since identical results were obtained in both regions, only the cerebellar experiments are presented here.
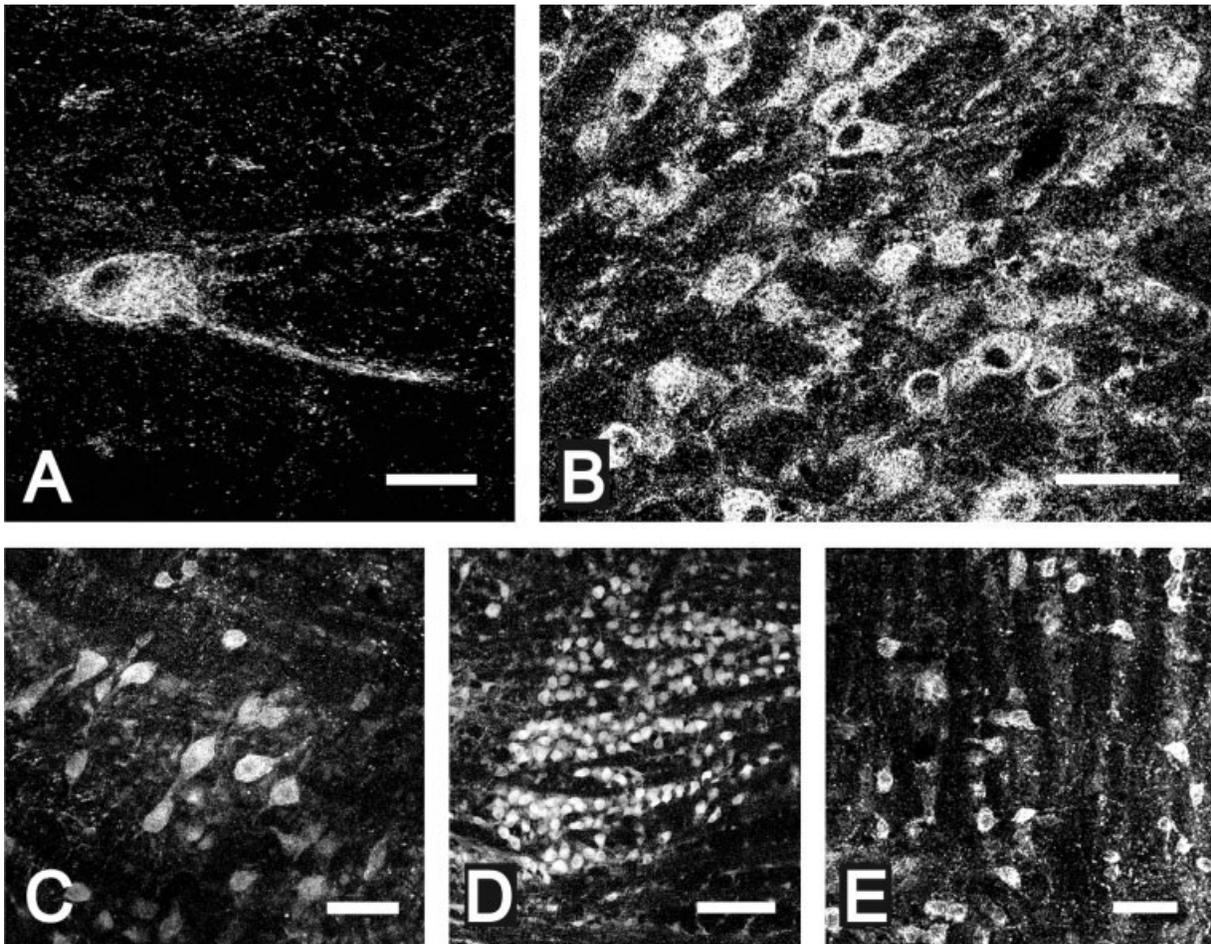
Fig. 2. Endogenous biotin is highly expressed in brainstem auditory nuclei. **A:** Spiral ganglion cells are positive for biotin. **B:** Several large-diameter neurons in the ventral cochlear nucleus are biotin-positive. **C:** Biotin was identified in somata and proximal dendrites of cells from the superior olivary nucleus. **D:** Biotin expression in cells of the MNTB was the highest of any nucleus within the brain. **E:** Neurons of the nucleus of the lateral lemniscus are positive for endogenous biotin. Label in A, B, and D was revealed by streptavidin-Cy3; C and E by antibiotin antibody and an AlexaFluor 488-conjugated secondary antibody. Scale bars = 50 μm.

We found that preabsorbing the antibiotin antibody with an excess of free biotin vanquished any label in cerebellar Purkinje cells (Fig. 4A,B). A commercially available streptavidin/biotin blocking kit was effective in eliminating streptavidin-Cy3 label (Fig. 4D,E). Likewise, a commercially available avidin/biotin blocking kit eliminated cell-specific HRP label indicated by the Vector Elite kit (Fig. 4G,H). We also found that 1 week postfixation of tissue slices in 2% PARA eliminated all labeling identified by any of the three cytochemical techniques (Fig. 4C,F,I), but not other immunogens (i.e., calbindin; data not shown). Finally, we found that when Triton X-100 was removed from our working solutions biotin could no longer be detected using streptavidin-Cy3 (Fig. 5A,B), the antibiotin antibody, or the Vector-Elite kit (not shown).

Nonspecific binding of avidin and streptavidin conjugates

Our experience in localizing endogenous biotin led us to consider potential difficulties arising from nonspecific binding by avidin and streptavidin conjugates, an issue that was most prominent with the Vector Elite kit. Although the kit consistently identified the same populations of biotin-positive neurons, there was a variable level of non cell-specific background. Interestingly, the nonspecific background label showed a pronounced rostral-caudal gradient. The gradient was characterized by a weak density of label in the forebrain (Fig. 5C,E,G) and a significantly greater density in the caudal brain (particularly the fiber tracts of the metencephalon and myelencephalon) (Fig. 5D,F,H). This gradient was evident, but only weakly, in sections treated with the antibiotin antibody (Fig. 5C,D) or with streptavidin-Cy3 (Fig. 5E,F), but was robust when using the Vector Elite kit (Fig. 5G,H).

Nonspecific binding of avidin to tissue is reportedly prevented by buffers of high ionic strength (e.g., 0.3–0.5 M NaCl) or alkaline pH (e.g., pH 9.4) (Guesdon et al., 1979; Clark et al., 1986; Jones et al., 1987). Hydrophobic and other protein–protein interactions can be blocked by adding 1–3% crystalline grade bovine serum albumin (BSA) or fetal calf serum to the working solution (Wood and Warnke, 1981). Binding to lectins is suppressed with
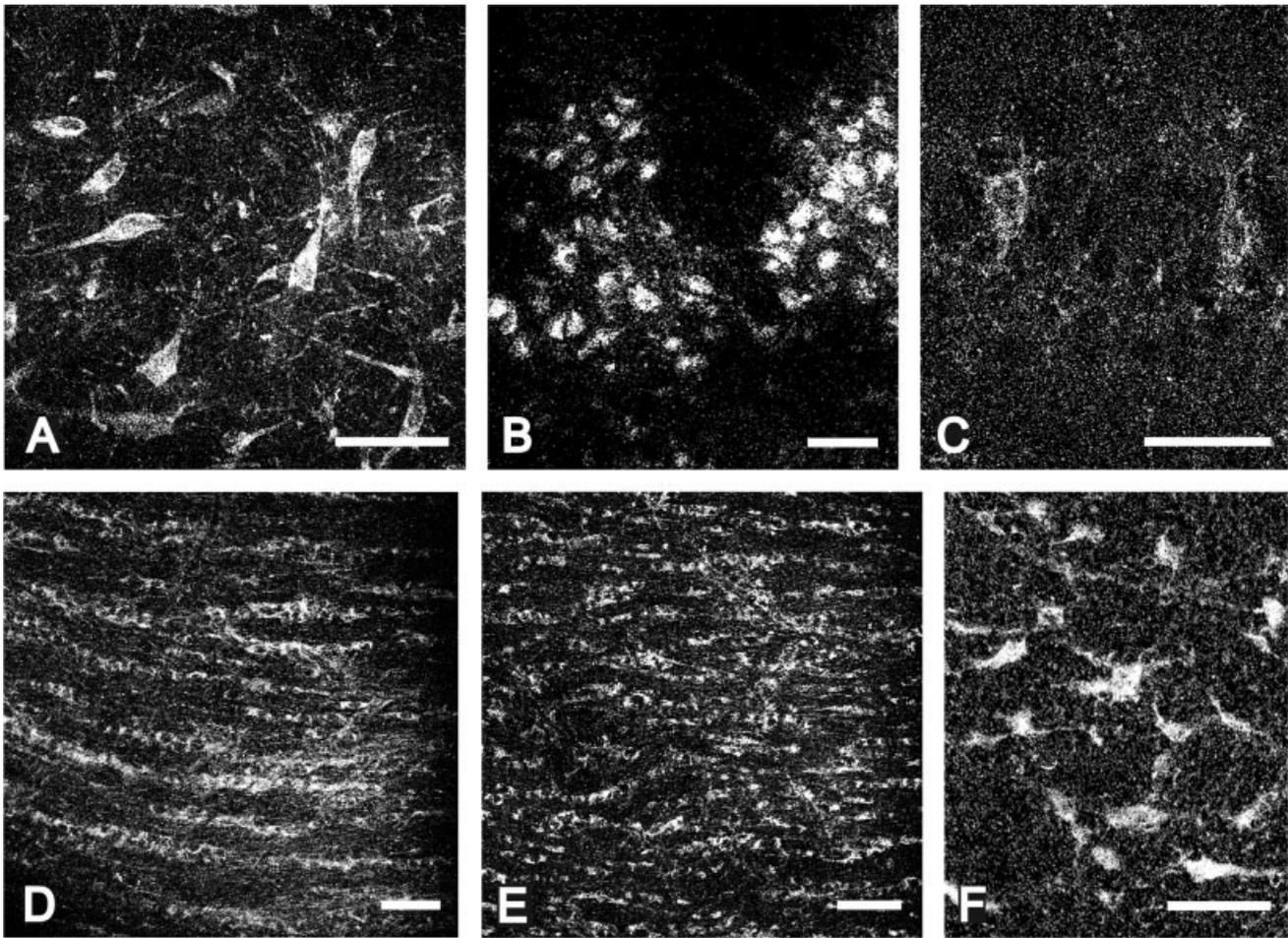
Fig. 3. Endogenous biotin is identified in only a few cell types of the rostral brain. **A:** Biotin staining was revealed in the somata and proximal dendrites of principal cells within the substantia nigra pars reticulata. **B:** Neurons of the lateral mammillary nucleus are biotin-positive. **C:** A limited number of hippocampal interneurons were weakly positive for endogenous biotin. **D–F:** Glial cells of the optic tract (D), anterior commissure (E), and spinal trigeminal tract (F) expressed detectable levels of biotin. Label in C–F was revealed by streptavidin-Cy3; A and B by anti-biotin antibody and a AlexaFluor 488-conjugated secondary antibody. Scale bars = 50 μm.

α-methyl-D-mannoside (Naritoku and Taylor, 1982). Commercially available blocking kits are based on the application of free avidin or streptavidin followed by free biotin to block all avidin or streptavidin binding sites. Notably, the sites blocked by these kits will include both endogenous biotin and any nonspecific avidin–tissue interactions. The blocking kits should then block cell-specific biotin label as well as background label indicated by either streptavidin conjugates (Cy3) or the avidin–biotin complex of the Vector Elite kit.

We implemented the following strategies to attempt to block the label indicated by the Vector Elite kit: 1) varying the ionic strength of the PB (0 M, 0.25 M, 0.5 M NaCl); 2) raising the pH of the buffer to 9.4; 3) including 1% BSA in the working solution; 4) preincubating sections in 0.05 M α-methyl-D-mannoside; and 5) applying streptavidin or avidin blocking kits. Preincubating in α-methyl-D-mannoside, including BSA in the working solution, or changing the ionic strength or pH of the buffer reduced but did not reliably eliminate all background label, with little effect on cell-specific biotin label. However, we noted that when HRP-labeled sections with low background were obtained, the PB was invariably of high ionic strength (0.5 M NaCl). The streptavidin blocking kit eliminated all labeling indicated by streptavidin-Cy3 (data not shown). In contrast, the avidin blocking kit eliminated all cell-specific labeling but only decreased the intensity of background label indicated by the Vector Elite kit. This latter result would suggest that some other element of the Vector Elite detection system not related to avidin binding sites also contributed to nonspecific labeling.

DISCUSSION

The present study describes detectable levels of biotin in select nuclei of rat CNS, with a prominent labeling of cell types in cerebellar motor and brainstem auditory nuclei. Biotin label was concentrated at the level of the brainstem, with virtually no label detected in cells rostral to the diencephalon. The distribution of biotin in cerebellar and auditory nuclei is particularly important, as it correlates
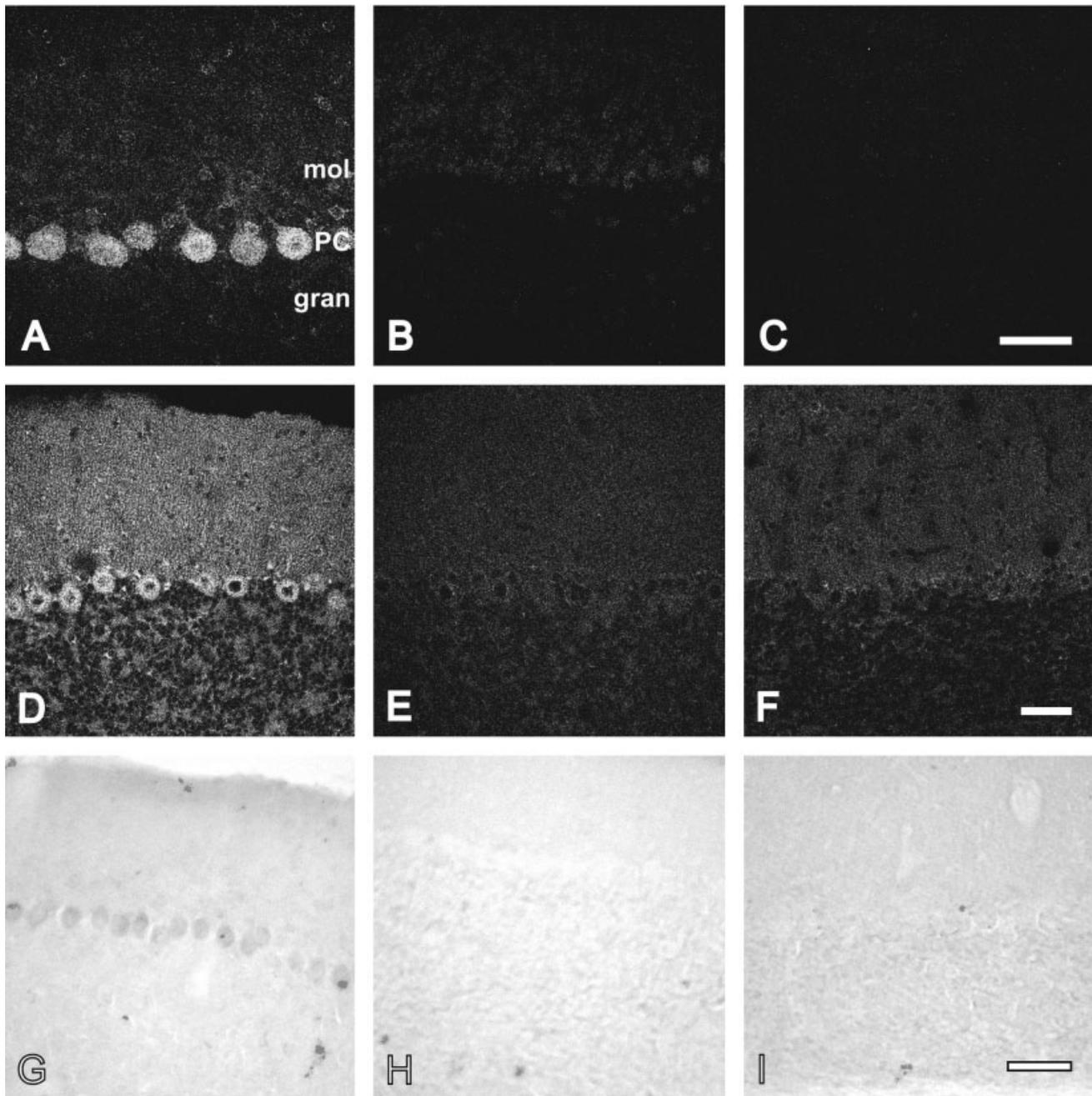
Fig. 4. Blocking endogenous biotin in cerebellar Purkinje cells. Shown are the results of three biotin-blocking methods for label identified with anti-biotin and AlexaFluor 488-conjugated secondary antibodies (A–C), streptavidin-Cy3 alone (D–F), or the Vector Elite kit alone (G–I). **B,E,H:** Preabsorption of antibiotin with excess free biotin (B), incubation with the streptavidin and biotin blocking kit (E) or incubation with the avidin and biotin blocking kit (H) all eliminated endogenous biotin labeling. **C,F,I:** One week postslice fixation of the tissue eliminated endogenous biotin labeling for all three techniques. PC, Purkinje cell layer; mol, molecular layer; gran, granule cell layer. Scale bars = 50 μm in C (applies to A–C); 50 μm in F (applies to D–F); 50 μm in I (applies to G–I).

with identified neurological symptoms that accompany disorders of biotin metabolism.

Biotin is selectively distributed in CNS

An endogenous expression of biotin was previously described in nonmammalian nervous systems. Biotin has been identified in the lobster giant axon (Ma, 1994) and in neurosecretory cells of the insect *Manduca sexta* (Ziegler et al., 1995). Biotin was identified in a subset of neurons from the turtle spinal cord (Berkowitz, 2002), in axons and synaptic boutons in numerous regions of frog brain (Eisner et al., 1996), in salamander and goldfish retina (Bhat-
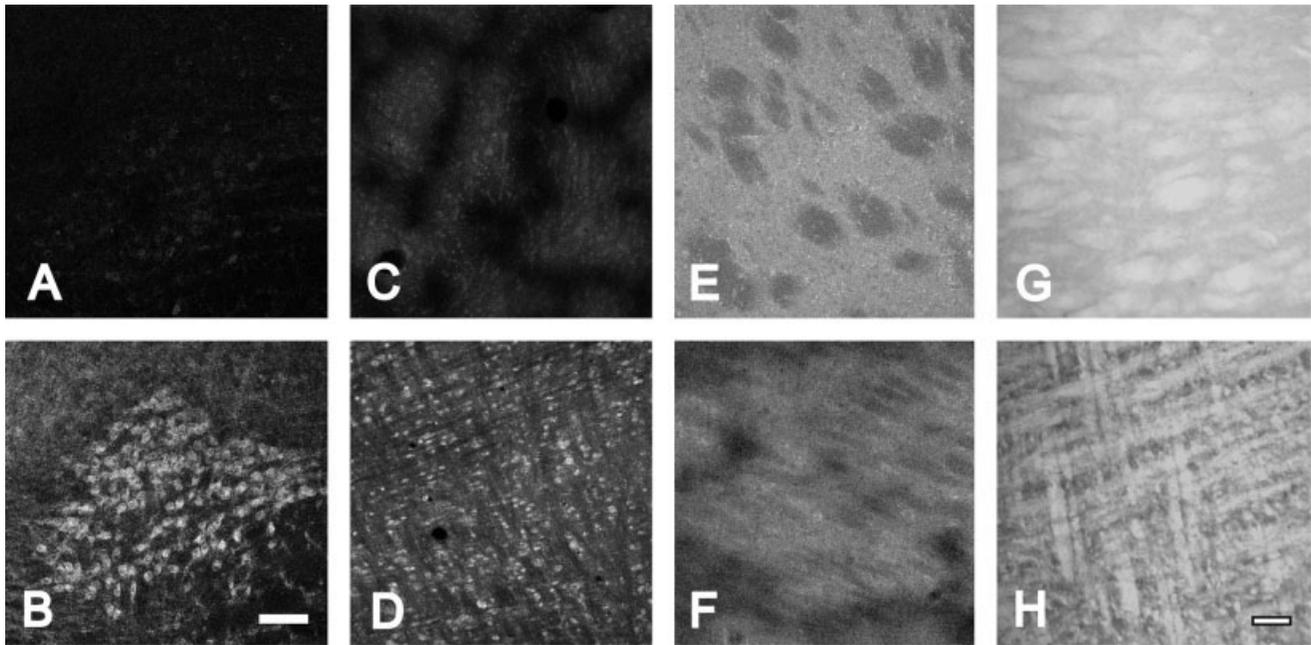
Fig. 5. **A,B:** Biotin label within the MNTB in the absence (A) and presence (B) of Triton X-100 in the working solution. **C–H:** Noncell associated background label is preferentially revealed in caudal brainstem. In sections from forebrain (striatum) only a weak background label is identified with the antibiotin antibody (C), streptavidin-Cy3 (E), or the Vector Elite kit (G). Low-power images of sections from caudal brainstem (myelencephalon) indicate a moderate amount of background label in sections labeled with antibiotin antibody (D) or streptavidin-Cy3 (F), while significant background label is noted with the Vector Elite kit (H). Scale bars = 100 μm in B (applies to A,B); 100 μm in H (applies to C–H).

tacharjee et al., 1997), and in two nuclei following song learning in birds (Johnson et al., 2000). Two earlier reports addressed the presence of endogenous biotin in the rat CNS, but only within a narrow region of forebrain spanning from the anterior hippocampus to anterior corpus callosum. These studies identified a few oligodendrocytes in tract systems (LeVine and Macklin, 1988) and intermittent labeling of neurons in hippocampus (Wang and Pevsner, 1999). Biotin labeling has also been reported in cerebellar Purkinje cells, although this was in pigs fed a biotin-supplemented diet (Cooper et al., 1997). These latter results are not easily compared with endogenous biotin in rats maintained on a normal diet, as artificial increases in biotin (as exemplified by ventricular injection of biocytin) leads to widespread labeling of cells that are ordinarily biotin-negative (McDonald et al., 1992; Ma, 1994).

We found a prominent but selective distribution of biotin in specific hindbrain nuclei, with highest levels of biotin in the cerebellar motor and auditory systems. This selective distribution is also supported by the localization of biotinidase, the degradative enzyme that cleaves biotin from biotinylated peptides in oxidative pathways. This enzyme has been localized through immunocytochemistry to a subset of CNS neurons that closely match our distribution of biotin (Heller et al., 2002). A preponderance of brainstem auditory nuclei are thus positive for biotinidase, including cells in the spiral ganglion, dorsal and ventral cochlear nuclei, and superior olivary nuclei, but not the inferior colliculus, medial geniculate, or cortical regions (Table 1). Northern blot analysis indicates high levels of biotinidase in cerebellum, although its cellular distribution in cerebellar cortex was not described. Labeling for biotinidase is evident in neurons of deep cerebellar nuclei and in the red nucleus. The correspondence between biotin and biotinidase was not complete, in that no labeling was reported for biotinidase in the MNTB or lateral lemniscus. Additional labeling for biotinidase was found in dendrites of hippocampal CA1 pyramidal cells, whereas biotin labeling in the present study was restricted to only a few interneurons. An additional correspondence to biotin labeling is apparent in the distribution of acyl-CoA-oxidase, a biotin-dependent oxidative enzyme (Farioli-Vecchioli et al., 2001). In this case, acyl-CoA-oxidase is expressed in cerebellar Purkinje cells, red nucleus, pontine nuclei, nucleus of the lateral lemniscus, and the substantia nigra pars reticulata.

Collectively, these studies indicate a selective distribution of biotin as well as enzymes implicated in the function and turnover of biotin in rat CNS. The reason for some discrepancies in the distribution of biotin and biotin-related enzymes is not fully known. However, since biotin labeling can vary depending on the conditions used (i.e., Triton X-100, duration of fix), the lack of full correspondence may simply reflect the prevailing conditions during immunoreactions.

Function of biotin

Biotin is an important molecule that is required for gluconeogenesis, fatty acid synthesis, and amino acid catabolism. It cannot be synthesized by mammals and must be derived from dietary intake or potentially through synthesis by gastrointestinal microflora (Wolf, 2001). 2D gel electrophoresis reveals the presence of at least 25 biotin-

containing proteins in mammalian tissues (Chandler and Ballard, 1985, 1986; Banks et al., 2003). Four of these principal bands have been described and correspond to pyruvate carboxylase, propionyl carboxylase, acetyl CoA carboxylase, and the α-subunit of methylcrotonyl-CoA carboxylase. Biotin is central to the function of these enzymes in acting as a CO_2 carrier during an ATP-dependent carbon fixation reaction. The identities of the remaining 20 plus bands have not been determined but likely match several members of an extensive list of biotin-containing proteins (Moss and Lane, 1971; Wood and Barden, 1977).

The vital importance of biotin is clearly shown by two autosomal recessive disorders that affect biotin levels: holocarboxylase synthetase deficiency and biotinidase deficiency. In the first case the ability of holocarboxylase synthetase to biotinylate carboxylases is disrupted, while in biotinidase deficiency the recycling of biotin is compromised and the level of free biotin is reduced. These conditions lead to multiple carboxylase deficiencies and development of clinical symptoms within months of birth, including ataxia, motor limb weakness, and hearing and visual loss (Sander et al., 1980; Wilcken and Hammond, 1983; Baumgartner and Suormala, 1997; Rahman et al., 1997; Wolf, 2001). It is thus significant that both biotin and biotinidase (Heller et al., 2002) are highly expressed in cells of the cerebellar motor system and brainstem auditory nuclei. Indeed, postmortem analyses of patients with biotinidase deficiency revealed chronic cerebellar degeneration characterized in part by a loss of the Purkinje cell layer and gliosis in the dentate nucleus, with further damage in the pons, medulla, and mammillary nuclei (Sander et al., 1980; Baumgartner and Suormala, 1997; Wolf, 2001). Our findings suggest that cells in each of these regions have a greater requirement for biotin, and thus enhanced susceptibility to disruptions of the biotin cycle. Although we are unaware of autopsy reports of damage to auditory nuclei in biotin disorders, the high level of biotin and biotinidase in auditory nuclei also suggests an increased susceptibility to biotin disruption and carboxylase deficiencies. A cellular correlate to visual loss and optic atrophy in biotin disorders is not as readily apparent, as neither biotin nor biotinidase can be detected in central visual nuclei. However, a report of endogenous biotin in retinal Müller cells identifies one potential site for damage to visual pathways (Bhattacharjee et al., 1997).

It is important to note that the approaches we used to detect biotin cannot discriminate between any of the biotin-containing proteins (Savage et al., 1992), and are thus expected to identify biotin in association with multiple functional roles. The idea that significant amounts of biotin might be concentrated in systems with similar functional purposes or energetic demands was explored by Ma (1994). That work demonstrated that significant concentrations of biotin are present in lobster giant axons, although in other species with giant axon escape systems biotin was not detectable (Ma, 1994). Higher levels of biotin expression in rat cerebellar motor and brainstem auditory nuclei may suggest a common metabolic requirement by these cell types. It is thus tempting to associate biotin expression with the high frequencies of spike discharge that are characteristic of several brainstem auditory cells, Purkinje cells, and hippocampal interneurons (Llinas and Sugimori, 1980; Lacaille and Williams, 1990; Morin et al., 1996; Martina et al., 1998; Wang et al., 1998).

Nevertheless, we also detected biotin in cells that exhibit comparatively lower frequencies of discharge (i.e., cerebellar nuclei) (Aizenman and Linden, 1999), indicating that this cannot be the only explanation. It was hypothesized that cells positive for biotin may have high needs for lipogenesis, as defined perhaps by a large membrane area to soma size ratio (e.g., a large, long axon and extensive dendritic arborizations coupled to a small soma). However, this was not supported by Ma (1994) or by the large variability in cell size of biotin-positive neurons in the present work. Other authors have suggested that biotin density correlates positively with increasing mitochondrial load (Bhattacharjee et al., 1997), although this assertion has not been verified at the level of electron microscopy. If cells have high oxidative requirements, and thus a greater density of biotin-containing carboxylases, it is apparent why a carboxylase deficiency and the associated acidosis could lead to cell death (Wolf, 2001). However, the presence of numerous biotin-containing proteins suggest that many potential roles for biotin remain to be determined.

Avidin–biotin technology for immunocytochemistry

The interaction between avidin–biotin or streptavidin–biotin is widely used in immunocytochemical reactions. The ABC technique is based on the extremely high affinity for binding between biotin and the egg-white protein avidin or the bacterial protein streptavidin (Hsu and Raine, 1981; Wilchek and Bayer, 1990). In fact, this interaction is three to six orders of magnitude greater than most antigen–antibody interactions, which underlies the enduring stability of the ABC complex (Green, 1975; Wilchek and Bayer, 1990). It is important to realize that the utility of the ABC technique relies solidly on either of two assumptions: that endogenous biotin is below the detection limit of the assay, or that endogenous biotin is present homogeneously throughout tissues and thus contributes only a uniform background label (Hsu and Raine, 1981; Wood and Warnke, 1981; Berkowitz, 2002). Endogenous biotin has been recognized as a potential source of false-positive labeling in immunocytochemistry and strategies for abolishing this label have been developed (Guesdon et al., 1979; Wood and Warnke, 1981; Hymes and Wolf, 2000). However, the potential for endogenous biotin to contribute to false-positive results in the CNS are often overlooked, likely due to the prevailing belief that endogenous biotin or biotinylated proteins are denatured during fixation (Hsu, 1990). We confirmed that prolonged periods of paraformaldehyde fixation (1 week) blocks detection of endogenous biotin. However, most immunocytochemical reactions do not (or cannot) incorporate such long time frames for fixation, leaving open the possibility of obtaining false-positive results when avidin or streptavidin conjugates are introduced. In most structures this would not present a problem, but it is a concern in the nuclear regions we found to express endogenous biotin. Fortunately, endogenous biotin is effectively blocked using commercial blocking kits (Fig. 4). The confounding effects of endogenous biotin can also be sidestepped entirely by not employing avidin or streptavidin conjugates and restricting immunocytochemical markers to secondary antibody conjugates.

An additional issue that became apparent in localizing biotin was the tendency for avidin or streptavidin conju-

gates to produce a diffuse and amorphic background label in different nuclei. This was especially the case for the avidin-HRP conjugate and reaction provided in the Vector Elite kit. The difference in intensity of background label indicated by avidin vs. streptavidin conjugates might relate to differences in the properties of these two molecules. Both avidin and streptavidin share several properties: they have similar molecular weights, both are tetramers with one biotin-binding site per subunit, and both display an equal affinity for biotin (Woolley and Longsworth, 1942; Chaiet and Wolf, 1964). However, streptavidin has a weakly acidic isoelectric point, moves as an anion during electrophoresis, and is non-glycosylated. Avidin has a strongly basic isoelectric point, moves as a cation during electrophoresis, and is rich in glycoproteins. The properties of avidin are then predicted to contribute to nonspecific interactions with anionic materials in neural tissues (Savage et al., 1992). A key potential source for nonspecific interactions are negatively charged glycosaminoglycans present within the extracellular matrix that are found in close association with the surface of cells (Hook et al., 1984; Celio et al., 1998; Yamaguchi, 2000). The exact composition of proteoglycans in the extracellular matrix also varies between brain regions (Yamaguchi, 2000), potentially accounting for higher levels of background label detected by avidin conjugates in specific brain nuclei. Our attempts to block this nonspecific label were only partially successful, and revealed additional background labeling by other molecular interactions most evident with the Vector Elite kit. The above results again emphasize the need to carefully consider the use of avidin or streptavidin conjugates in immunocytochemical procedures.

In summary, we have demonstrated that detectable levels of biotin are distributed selectively in the rat CNS. Biotin is highly expressed in the cerebellar motor and brainstem auditory systems, providing a cellular correlate to neurological symptoms associated with multiple carboxylase deficiencies. The presence of endogenous biotin has further implications for immunochemical studies in specific nuclei. In these regions the use of avidin or streptavidin conjugates should be avoided or appropriate steps taken to eliminate potential false-positive results arising from endogenous biotin.

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