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Short communication

Endogenous biotin staining in a subset of spinal neuronal cell bodies: a potential confounding factor for neuroanatomical studies

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Abstract

Biotinylated compounds are commonly used to label neuronal cell bodies via intracellular filling or retrograde tracing. Endogenous concentrations of biotin within a subset of neuronal cell bodies would pose a problem for interpreting such experiments. Here I report that a subset of turtle spinal cord neuronal cell bodies strongly stains for biotin, using the avidin–biotin–horseradish peroxidase (ABC) reaction, in the absence of any exogenous biotinylated compound.

Theme: Cellular and molecular biology

Topic: Staining, tracing, and imaging techniques

Keywords: ABC; Avidin; Biotin; Biocytin; Neurobiotin; Biotinylated dextran amine

Biotinylated compounds such as biocytin, neurobiotin, and biotinylated dextran amine have become important tools to study the locations and morphologies of neurons [9,11,12,15,18,22–24,27]. These biotinylated compounds can be introduced by intracellular injection of single neurons [9,15,22] or by retrograde labeling of neuronal populations [14,15,18,23,24,27]. Biotin can be visualized via its highly specific binding to the egg-white protein, avidin [7], which can be coupled to a fluorescent molecule or to an enzyme that reacts with a chromagen (e.g. the avidin–biotin-complex, or ABC reaction) [8,10,26]. These uses of exogenous biotin rely on the tacit assumption that endogenous biotin either exists at too low a level to be detected with the techniques employed or produces merely a light and uniform background staining of all cells.

Biotin (vitamin H) is a coenzyme obtained from the diet and used by several enzymes to transfer carboxyl groups [5,16]. Thus, biotin (or its lysine-coupled form, biocytin, which also occurs naturally [5,21]) might be concentrated in particular cell types, depending on their metabolic needs. In fact, previous research has shown that endogenous biotin staining can be concentrated in rat brain neurons [28] and oligodendrocytes [19], bird brain tissue [13], frog brain axons and synaptic boutons [6], and specific subtypes of neuronal cell bodies in salamander and goldfish retinas [4] and lobster nerve cord [20]. In addition, exogenous biocytin injected into a rat brain ventricle becomes concentrated in particular types of neuronal cell bodies [21]. The current study reports that endogenous biotin staining can also be concentrated in a subset of neuronal cell bodies in the spinal cord of a reptile.

The dorsal 7 (D7) spinal segment through the sacral 2 (S2) segment were exposed in anesthetized adult turtles (*Trachemys scripta elegans*), obtained in May from a supplier in Louisiana (Candy's Quality Reptiles, Laplace, LA). Surgical methods have been described previously [2]. Animals deeply anesthetized with sodium pentobarbital (1.0 ml of 390 mg/ml solution, i.p.) were perfused transcardially with 600–800 ml turtle saline containing 0.1% sodium nitrite and 10 units/ml sodium heparin, followed by 200 ml cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The D7–S2 spinal cord, which includes the entire hindlimb enlargement (D8–S2), was postfixed at 4 °C for 16–18 h and then placed in 20% sucrose/PB at 4 °C for 3–5 h. The spinal cord was then placed in an embedding medium containing 0.25% gelatin, 20% powdered egg albumin, and 10% sucrose in PB for 30–60 min at 4 °C; the embedding medium was hardened

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by addition of glutaraldehyde to a final concentration of 2.5%.

The tissue block was frozen and sectioned horizontally on a sliding microtome at 50 μm. Sections were rinsed in PB three times (5 min each), incubated 20 min in 0.3% hydrogen peroxide/PB to reduce endogenous peroxidase staining, rinsed four times in PB (10 min each), and incubated 16–18 h in 1:100 ABC solution (Vectastain Elite Standard ABC Kit, PK-6100, Vector Laboratories, Burlingame, CA)/PB (i.e. one drop of each ABC reagent/5 ml PB) with 0.3% Triton X-100, all at 4 °C on a rotator. This concentration of ABC reagents is one-half the concentration recommended by Vector. Sections were rinsed four times (10 min each) in PB on a rotator and then divided into two groups, with alternate sections reacted with different chromagens. One group was pre-incubated 20 min in 0.05% diaminobenzidine (DAB; Sigma, St. Louis, MO), 0.025% cobalt chloride, and 0.02% nickel ammonium sulfate in 0.05 M phosphate buffer, pH 7.4; then hydrogen peroxide was added to a final concentration of 0.005% and the sections were reacted for 25 min; sections were rinsed six times (5 min each) in PB [1]. The other group was instead incubated in 1:67 VIP solution (VIP Substrate Kit for Peroxidase, SK-4600, Vector Laboratories) in PB (i.e. three drops of each reagent/10 ml PB; this is one-half the concentration recommended by Vector) for 7–10 min; sections were rinsed three times (10 min each) in PB. Both reactions were conducted at room temperature on a rotator. Sections were mounted with Albrecht's solution (0.75% gelatin in 40% ethanol) on gelatin-coated slides, air-dried, dehydrated in ethanols, cleared in xylenes, and cover-slipped [3].

Cell bodies were counted as labeled neuronal cell bodies only if: (1) they were much more darkly labeled than any background staining, and (2) they included both a stained cell body and an attached stained process visible in the same section. Photomicrographs were acquired using a digital camera; they were rotated, arranged, and labeled, and Fig. 1F was uniformly lightened, using Adobe Photoshop, but photomicrographs were not otherwise manipulated.

A subset of spinal cord neuronal cell bodies was darkly stained, against a background of unstained or lightly stained gray matter, and the stained neurons were concentrated in particular spinal cord regions. Staining was concentrated in the cytoplasm (Fig. 1). Darkly stained neuronal cell bodies were consistently seen in the ventral horn, especially in its ventral half (Fig. 1A–E). (In the turtle spinal cord, laminae are not clearly distinguishable [17].) These cell bodies were typically large, 30–60 μm in diameter, and multipolar or bipolar. Multiple proximal dendrites were often stained. A few cells had a size, morphology, and location consistent with their being motoneurons [3,25], but most did not. Darkly stained ventral horn neurons were seen in each of the six spinal segments examined in each of two animals (Fig. 2).

A second population of stained neuronal cell bodies was seen in the intermediate zone of the D7 segment (Fig. 1F, G and 2). These cells occurred in clusters near the dorsoventral level of the central canal (Fig. 1F and G). They were relatively homogeneous in size and morphology, ~25 μm in diameter and multipolar. These cells were generally more lightly stained than the stained ventral horn neurons (e.g. compare Fig. 1F and G to Fig. 1A–E). This population of stained intermediate zone cells was only seen in one of the two animals examined (Fig. 2), perhaps because the amount of endogenous biotin present was just below the threshold of detection in the other spinal cord processed. Virtually no labeled neuronal cell bodies were seen in the dorsal horn of either animal (Fig. 2).

Labeled neuronal cell bodies were seen with both DAB and VIP reactions, indicating that the staining is not an artifact of one chromagen. To test the hypothesis that stained neurons contain endogenous concentrations of peroxidase, the sectioned spinal cord of an additional animal was reacted with VIP, without a preceding ABC reaction. No labeled neurons were seen. To test the hypothesis that the stained neurons contain endogenous concentrations of biotin or avidin, the sectioned spinal cord of an additional animal was reacted with 1:1 avidin solution in PB, followed by 1:1 biotin solution in PB (Blocking Kit, SP-2001, Vector Laboratories), to block all binding sites on endogenous biotin or avidin; ABC and VIP reactions were then carried out. No labeled neurons were seen.

To test for any effect of the embedding medium (which contained egg albumin and therefore avidin), the D7–S2 spinal cord of one additional animal was instead embedded in warmed 12% gelatin/10% sucrose/PB; the block was cooled for 4 min at −20 °C, fixed in 4% paraformaldehyde/2.5% glutaraldehyde/PB for 30 min at 4 °C, placed in 20% sucrose/PB for 40 min at 4 °C, and then frozen-sectioned and processed with ABC and VIP reagents as described above. Animals at this point were obtained in December from an Oklahoma supplier (Millard's Turtles, Holdenville, OK), so for comparison a second spinal cord from the same group of animals was embedded in the standard gelatin/albumin/sucrose medium and processed with ABC and VIP reagents as above. The spinal cord embedded in gelatin/sucrose contained three clearly stained neuronal cell bodies and the cord embedded in gelatin/albumin/sucrose contained five clearly stained neuronal cell bodies, all in the ventral horn; in addition, the latter cord contained 20–30 lightly labeled neuronal cell bodies in the D7 intermediate zone. Thus, clearly stained neuronal cell bodies were seen in similar numbers and locations with either embedding medium and therefore were not artefacts of the embedding medium. The total number of stained neuronal cell bodies, however, was much lower in turtles obtained from Oklahoma in December than in turtles obtained from Louisiana in May. These differences might be due to seasonal, dietary, or

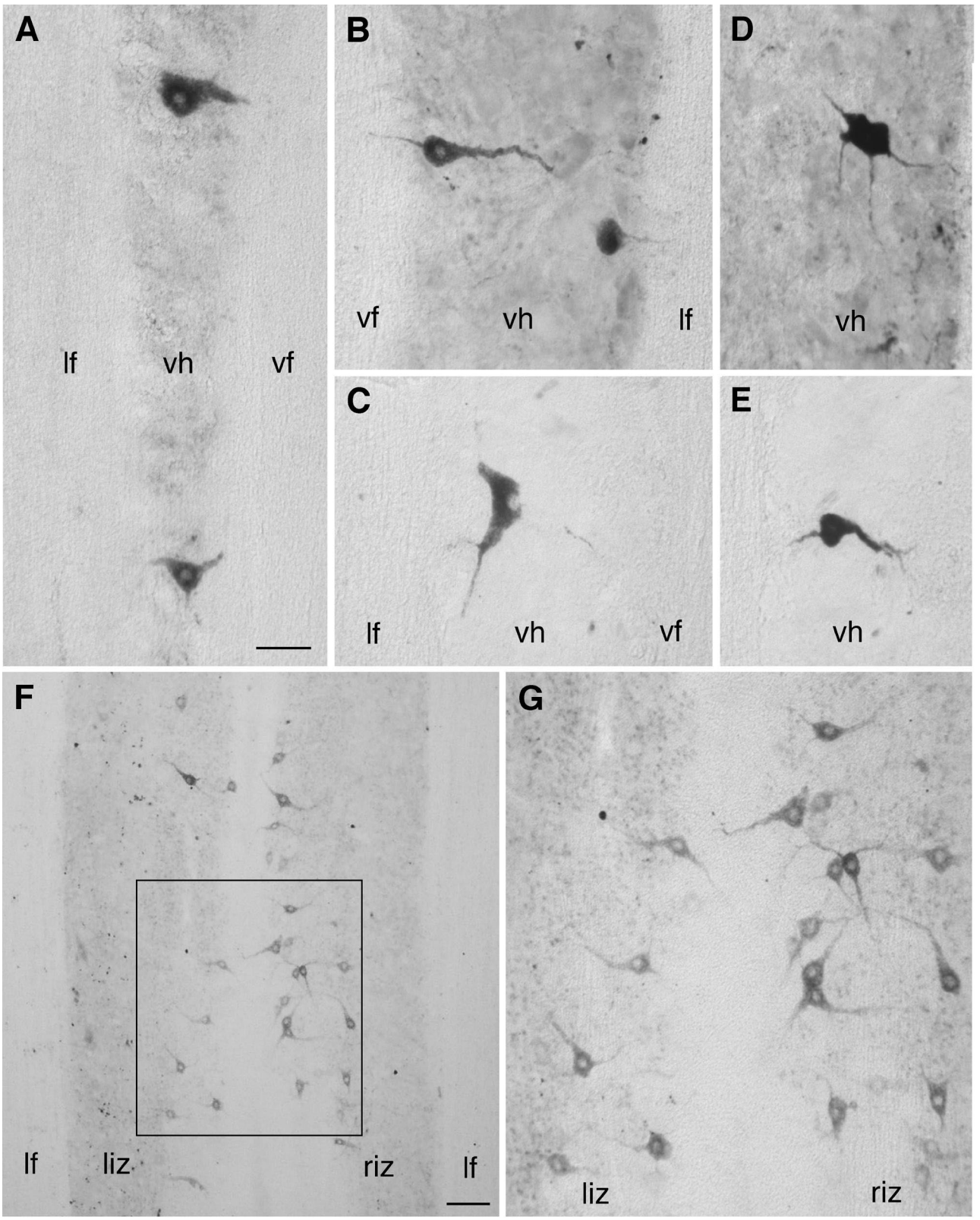

Fig. 1. Photomicrographs showing that endogenous biotin staining is concentrated in a subset of spinal cord neuronal cell bodies. (A–E) Stained ventral horn neurons and (F, G) stained intermediate zone neurons; (G) shows the boxed region of (F) at higher magnification. All sections are horizontal, through the D7 (F, G), D8 (B), D9 (C, E), S1 (D), and S2 (A) spinal cord segments. Rostral is up in each panel; the scale bar in (A) is 50 μm and applies to (A–E) and (G); the scale bar in (F) is 100 μm. DAB was the chromagen for (D); VIP was the chromagen for (A–C) and (E–G). (C) and (E) are from one animal and (A, B, D) and (F, G) are from another animal. lf, lateral funiculus; liz, left intermediate zone; riz, right intermediate zone; vf, ventral funiculus; vh, ventral horn.

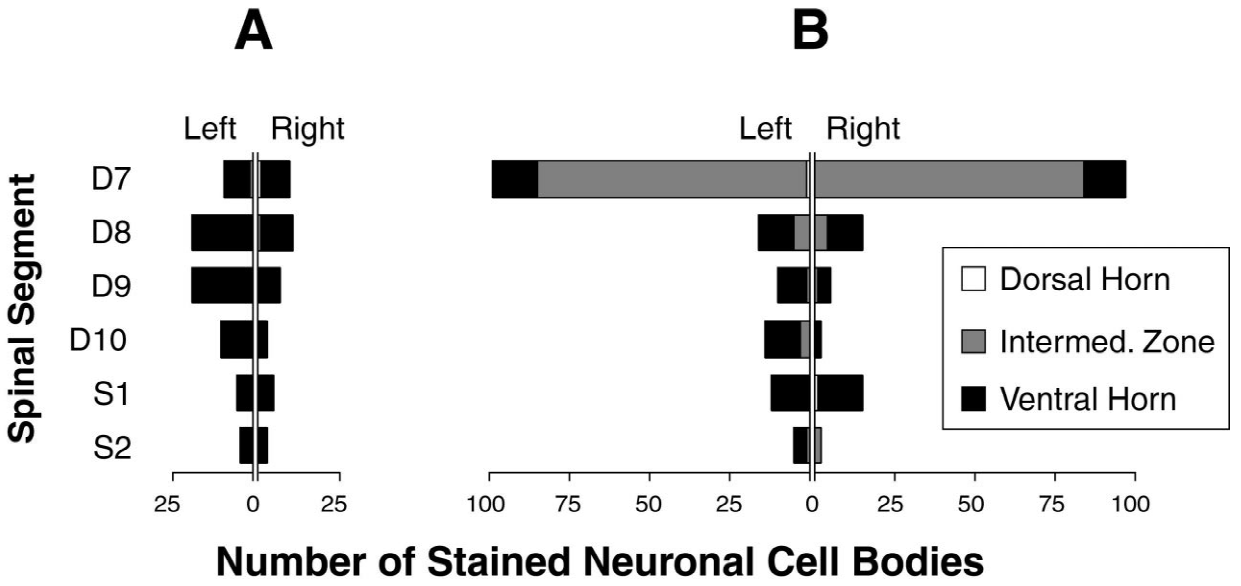

Fig. 2. Distribution of neuronal cell bodies showing endogenous biotin staining in the D7–S2 spinal cord segments of two animals (A and B).

other environmental factors. Previous research on larger numbers of frogs and toads has shown that endogenous neuronal biotin staining can be either strong and abundant or weak and sparse, depending on the animal supplier and related environmental factors [6].

These findings are consistent with the hypothesis that a subset of spinal cord neurons endogenously concentrates a substance that specifically binds avidin or biotin. This substance is most likely biotin or biocytin, both of which can be found naturally in adult cells [5,21]. The regional concentration of stained neuronal cell bodies suggests that particular subtypes of neurons contain higher concentrations of endogenous biotin or biocytin, perhaps due to special metabolic needs [6,21].

Previous research has shown that biotin staining can be naturally concentrated within subsets of neuronal cell bodies in the brains of rats [28] and birds [13], the retinas of salamanders and fish [4], and the nerve cord of arthropods [20], as well as in amphibian brain neuronal processes [6] and rat brain glial cells [19]. The current findings show that biotin staining can also be naturally concentrated within a subset of neuronal cell bodies in the spinal cord and in a reptile. Thus, experiments in which an exogenous biotinylated compound is introduced into the nervous system and visualized using an ABC reaction may lead to staining of some neuronal cell bodies that had not been exposed to the exogenous biotin, in addition to cells that were deliberately labeled, in a wide variety of species and parts of the nervous system. If thousands of neuronal cell bodies are labeled exogenously via a large extracellular injection and only a few hundred neurons are labeled endogenously (as in the turtle spinal cord), the endogenous biotin staining may only slightly confound interpretation of the results. If, however, a relatively small number of neuronal cell bodies are exogenously labeled, via either intracellular fills or a small extracellular injection, endogenously labeled cells could be a major confounding factor. The extent to which endogenous biotin staining of neuronal cell bodies occurs in additional species and additional parts of the nervous system remains to be seen. Therefore, for each type of nervous system tissue studied, it will be important to perform control reactions on tissue that has not been exposed to an exogenous biotinylated compound.

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References

[1] J.C. Adams, Heavy metal intensification of DAB-based HRP reaction product, J. Histochem. Cytochem. 29 (1981) 775.
[2] A. Berkowitz, Broadly tuned spinal neurons for each form of fictive scratching in spinal turtles, J. Neurophysiol. 86 (2001) 1017–1025.
[3] A. Berkowitz, P.S.G. Stein, Descending propriospinal axons in the turtle hindlimb enlargement: cells of origin and funicular courses, J. Comp. Neurol. 346 (1994) 321–336.
[4] J. Bhattacharjee, B. Nunes Cardozo, W. Kamphuis, M. Kamermans, G.F. Vrensen, Pseudo-immunolabelling with the avidin–biotin–peroxidase complex (ABC) due to the presence of endogenous biotin in retinal Muller cells of goldfish and salamander, J. Neurosci. Methods 77 (1997) 75–82.
[5] K. Dakshinamurti, J. Chauhan, Biotin, Vitam. Horm. 45 (1989) 337–384.

[6] S. Eisner, E. Harris, S.M. Galoyan, J.Y. Lettvin, F. Scalia, Concentration and storage of biotin in the amphibian brain, J. Comp. Neurol. 368 (1996) 455–466.
[7] P. Gyorgy, The curative factor (vitamin H) for egg white injury, with particular reference to its presence in different foodstuffs and in yeast, J. Biol. Chem. 131 (1939) 733–744.
[8] H. Heitzmann, F.M. Richards, Use of the avidin–biotin complex for specific staining of biological membranes in electron microscopy, Proc. Natl. Acad. Sci. 71 (1974) 3537–3541.
[9] K. Horikawa, W.E. Armstrong, A versatile means of intracellular labeling: injection of biocytin and its detection with avidin conjugates, J. Neurosci. Methods 25 (1988) 1–11.
[10] S.M. Hsu, L. Raine, H. Fanger, Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures, J. Histochem. Cytochem. 29 (1981) 577–580.
[11] M.F. Jacquin, J.W. Hu, B.J. Sessle, W.E. Renehan, P.M. Waite, Intra-axonal neurobiotin injection rapidly stains the long-range projections of identified trigeminal primary afferents in vivo: comparisons with HRP and PHA-L, J. Neurosci. Methods 45 (1992) 71–86.
[12] X. Jiang, R.R. Johnson, A. Burkhalter, Visualization of dendritic morphology of cortical projection neurons by retrograde axonal tracing, J. Neurosci. Methods 50 (1993) 45–60.
[13] F. Johnson, E. Norstrom, K. Soderstrom, Increased expression of endogenous biotin, but not BDNF, in telencephalic song regions during zebra finch vocal learning, Dev. Brain Res. 120 (2000) 113–123.
[14] M.A. King, P.M. Louis, B.E. Hunter, D.W. Walker, Biocytin: a versatile anterograde neuroanatomical tract-tracing alternative, Brain Res. 497 (1989) 361–367.
[15] H. Kita, W. Armstrong, A biotin-containing compound *N*-(2-aminoethyl)biotinamide for intracellular labeling and neuronal tracing studies: comparison with biocytin, J. Neurosci. Methods 37 (1991) 141–150.
[16] J.R. Knowles, The mechanism of biotin-dependent enzymes, Annu. Rev. Biochem. 58 (1989) 195–221.
[17] A. Kusuma, H.J. ten Donkelaar, R. Nieuwenhuys, Intrinsic organization of the spinal cord, in: C. Gans, R.G. Northcutt, P. Ulinski (Eds.), Biology of the Reptilia, Vol. 10, Academic Press, New York, 1979, pp. 59–109.
[18] S.R. Lapper, J.P. Bolam, The anterograde and retrograde transport of neurobiotin in the central nervous system of the rat: comparison with biocytin, J. Neurosci. Methods 39 (1991) 163–174.
[19] S.M. LeVine, W.B. Macklin, Biotin enrichment in oligodendrocytes in the rat brain, Brain Res. 444 (1988) 199–203.
[20] P.M. Ma, Biotin staining in the giant fiber systems of the lobster, J. Comp. Neurol. 341 (1994) 567–579.
[21] A.J. McDonald, F. Mascagni, Y.D. Riley, R.L. Neal, M. Brinley-Reed, Biocytin injections produce selective neuronal labeling in the rat CNS, Neuroreport 3 (1992) 337–340.
[22] M. Pu, D.M. Berson, A method for reliable and permanent intracellular staining of retinal ganglion cells, J. Neurosci. Methods 41 (1992) 45–51.
[23] N. Rajakumar, K. Elisevich, B.A. Flumerfelt, Biotinylated dextran: a versatile anterograde and retrograde neuronal tracer, Brain Res. 607 (1993) 47–53.
[24] A. Reiner, C.L. Veenman, L. Medina, Y. Jiao, N. Del Mar, M.G. Honig, Pathway tracing using biotinylated dextran amines, J. Neurosci. Methods 103 (2000) 23–37.
[25] T.J.H. Ruigrok, A. Crowe, D.H.J. ten, Morphology of lumbar motoneurons innervating hindlimb muscles in the turtle *Pseudemys scripta elegans*: an intracellular horseradish peroxidase study, J. Comp. Neurol. 230 (1984) 413–425.
[26] J. Sipos, B.M. McFarlane, C.G. McSorley, C.D. Gove, R. Williams, I.G. McFarlane, Immunohistochemical demonstration of the asialoglycoprotein receptor in rat liver by a sensitive avidin–biotin technique, J. Pathol. 158 (1989) 247–252.
[27] N. Todorova, G.S. Rodziewicz, Biotin–dextran: fast retrograde tracing of sciatic nerve motoneurons, J. Neurosci. Methods 61 (1995) 145–150.
[28] H. Wang, J. Pevsner, Detection of endogenous biotin in various tissues: novel functions in the hippocampus and implications for its use in avidin–biotin technology, Cell. Tissue Res. 296 (1999) 511–516.