ELSEVIER

Journal of Neuroscience Methods 99 (2000) 37–44

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

Quantitation of tyrosine hydroxylase protein in the locus coeruleus from postmortem human brain

Meng-Yang Zhu [a], Violetta Klimek [a], John W. Haycock [c], Gregory A. Ordway [a,b,*]

[a] *Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA*

[b] *Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS 39216-4505, USA*

[c] *Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA, USA*

Received 14 May 1999; received in revised form 17 March 2000; accepted 22 March 2000

Abstract

In this study, we developed an immuno-autoradiographic method to obtain quantitative estimates of tyrosine hydroxylase (TH) protein in tissue sections from post-mortem human brain. Protein from tissue sections containing the locus coeruleus (LC) was directly transferred to a polyvinylidene fluoride (PVDF) membrane. Immunoreactive TH on PVDF membranes was identified with optimized concentrations of TH antibody followed by application of [^{125}I]labeled secondary antibody. Quantities of TH on autoradiograms were estimated by comparing optical densities of transferred immunoblots to a calibrated standard curve produced with purified recombinant TH dotted onto the same PVDF membranes. Amounts of TH-immunoreactivity in the LC were proportional to the thickness of tissue sections up to 15 μm. However, the amounts of total protein, as measured by Ponceau S staining, were linearly related to section thicknesses up to 30 μm. Comparisons of quantities of immunoreactive TH in the LC using this method to amounts determined using traditional Western blotting, in which LC tissue was punched from adjacent sections from the same subject, showed a positive correlation ($r^2 = 0.99$, $P < 0.01$). Using the transfer immunoblot method, an uneven distribution of TH protein was observed along the rostrocaudal axis of the human LC ($P < 0.01$). This method may provide a sensitive and useful tool for the study of the role of human TH expression in the pathophysiology of psychiatric disease.

Keywords: Tyrosine hydroxylase; Locus coeruleus; Norepinephrine neuron; Immunoblotting

1. Introduction

Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase) catalyzes the initial and rate-limiting step in catecholamine biosynthesis (Nagatsu et al., 1964; Levitt et al., 1965). The expression of TH in the brain is regulated by physiological and pharmacological factors, including drugs (Reis et al., 1974; Zigmond, 1979; Nestler et al., 1990), nerve growth factors (Thoenen et al., 1971) and is affected by stress (Thoenen, 1970). Changes in TH expression reflect changes in the activity of noradrenergic neurons of the locus coeruleus (LC), (for review, see Zigmond et al., 1989). Factors that increase the firing activity of these neurons induce an increase in the concentration of TH in the LC (Melia and Duman, 1991; Melia et al., 1992). In contrast, factors that decrease the firing rate of these neurons, such as tricyclic antidepressants, decrease TH in the LC (Melia et al., 1992). Hence, measurement of TH in the LC, postmortem, may yield important information regarding the pre-mortem activity of central noradrenergic neurons.

Measurement of TH in the LC from post-mortem tissue of subjects with psychiatric illnesses may provide important information relative to the pathophysiology of catecholaminergic systems in these disorders. This is of particular interest in the study of diseases, such as anxiety, depression and schizophrenia, that have been postulated to involve changes in noradrenergic activity (Prange, 1964; Redmond and Huang, 1979; Lake et al., 1980; Kostowski et al., 1986). Previously, we used

* Corresponding author. Tel.: +1-601-9845893; fax: +1-601-9845894.

E-mail address: gordway@psychiatry.umsmed.edu (G.A. Ordway).

0165-0270/00/$ - see front matter
PII: S0165-0270(00)00211-9

quantitative Western blotting (Ordway et al., 1994) to measure TH in the LC from victims of suicide and control subjects. In this method, the LC is dissected and punched out from a slice of pontine tissue, then solubilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). While this technique provides a relatively accurate determination of the amount of TH protein in the tissue section or punch containing the LC, its limitation is a lack of anatomical resolution, given the very small size of the LC relative to the size of the total amount of tissue that can be reliably dissected. Since TH concentrations would be expected to decrease with distance from the immediate cellular region of the LC, the diameter of the punch or total size of the dissected tissue section would be expected to greatly influence the concentrations of TH measured by Western blotting. Furthermore, deviations in the dissection (punch) of the LC could also contribute to variability of measurements of TH concentrations. The establishment of immunoautoradiography directly in unfixed sections of rat brain (Raisman-Vozari et al., 1991) or in frozen tissue sections of rat brain transferred to nitrocellulose (Weissmann et al., 1989), has permitted quantitative analysis of TH with retention of anatomical specificity. This technique could prove to be useful in the measurement TH in human tissue post-mortem. In this report, we describe a similar immuno-autoradiographic technique to measure quantitatively TH in the LC in tissue sections from post-mortem human brain.

2. Materials and method

2.1. Tissue collection and dissection

Human brain tissue was collected from the Cuyahoga County Coroner's Office, Cleveland, OH, in accordance with an approved Institutional Review Board protocol for human studies. Subjects were coded to protect their identity. Cause of death was determined by the Coroner. On arriving at the Coroner's office, all subjects were kept in a refrigerated room prior to autopsy. The LC of each subject was sectioned (thickness as specified) at -16°C with a cryostat microtome sequentially throughout its entire length beginning at its rostral end (at the level of the frenulum). Tissue sections were thaw-mounted onto gelatin-coated microscope slides and then dried under a stream of air at room temperature, dessicated and stored at -80°C until assay.

2.2. Section transfer

Frozen slide-mounted tissue sections were removed from the freezer and placed under a stream of air to warm to room temperature and dry. Polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA) were wetted with methanol, rinsed with Tris/10% methanol buffer (pH 7.4), then placed on filter paper which had been soaked with same buffer. The slides (tissue side down) were carefully placed onto the membranes, marking the beginning of tissue transfer. The transfer was stopped by removing the slide from the membranes. Extra caution was taken in order to avoid smearing the slide across the membrane during removal. Tissue sections are retained on the slide and do not adhere to the membrane. Membranes were submerged in Tris-buffered saline (TBS) with gentle shaking for 10 min, followed by submersion in TBS/0.1% Tween-20 for 20 min.

2.3. Immunoblotting

Membranes were pre-incubated in a blocking solution (10% non-fat dried milk in TBS with 0.1% Tween-20, 2 h at room temperature), then washed three times with TBS/0.1% Tween-20. Membranes were incubated with primary antibody (mouse anti-TH, 1:20 000 dilution, 4°C) overnight with gentle shaking and then incubated with secondary [^{125}I]labeled anti-mouse whole antibody from sheep (1:200 dilution, Amersham, Arlington Heights, IL) for 2 h at room temperature followed by washing. Membranes were apposed to [^{3}H]-sensitive Ultrofilm (Amersham) and exposed for 6–10 days. Optical densities of developed autoradiograms were quantified using an image analysis system (MCID M2; Imaging Research Inc., Ont., Canada).

2.4. Calibration

TH standards (eight concentrations) were made by mixing purified recombinant rat TH with a homogenate of human cerebellum prepared in a sodium dodecyl sulfate (SDS, 1%) solution containing 2 mM EDTA (pH 8). Protein concentrations of human cerebellum preparation were measured by a modified method of Lowry (Peterson, 1977) and adjusted so that the total protein concentration (TH protein plus human cerebellar protein) of each standard was 0.5 μg/μl. An aliquot (1 μl) of each standard was spotted onto the same PVDF membrane on which the tissue section was transferred. In certain experiments, the brain tissue-transferred PVDF membranes were air-dried and stained with Ponceau S (overnight). Membranes were destained with a 5% acetic acid solution to reduce the non-specific staining by dye and dried again. Protein amounts on the membranes were quantitated (MCID M2) using a set of calibrated cerebellar protein standards that were spotted onto the same membrane and stained simultaneously.

2.5. Western blotting studies

The LC and a small amount of surrounding tissue was punched (5 mm diameter punch) from tissue sections cut transversely through a block of pontine brainstem containing the LC. The punched LC tissues were placed in Eppendorf tubes and were lysed in a warmed 1% SDS solution containing 2 mM EDTA (pH 8). Tissues were sonicated twice (10 s each) and solubilized samples were heated in a water bath at 100°C for 5 min. Equal protein aliquots were loaded onto SDS-polyacrylamide gels (7.5% gel, 1.5 mm) for electrophoresis (PAGE). Proteins in gels were electro-transferred (35 V, 16 h, 4°C) to PVDF membranes. The blots were incubated with primary and [^{125}I]secondary antibodies followed by washing as described above. Immunolabeling was visualized by autoradiography and quantified by an image analysis system (MCID M2) using the calibration curves generated from a set of purified recombinant rat TH standards, which were mixed with rat liver carrier protein (to adjust for equal protein loads).

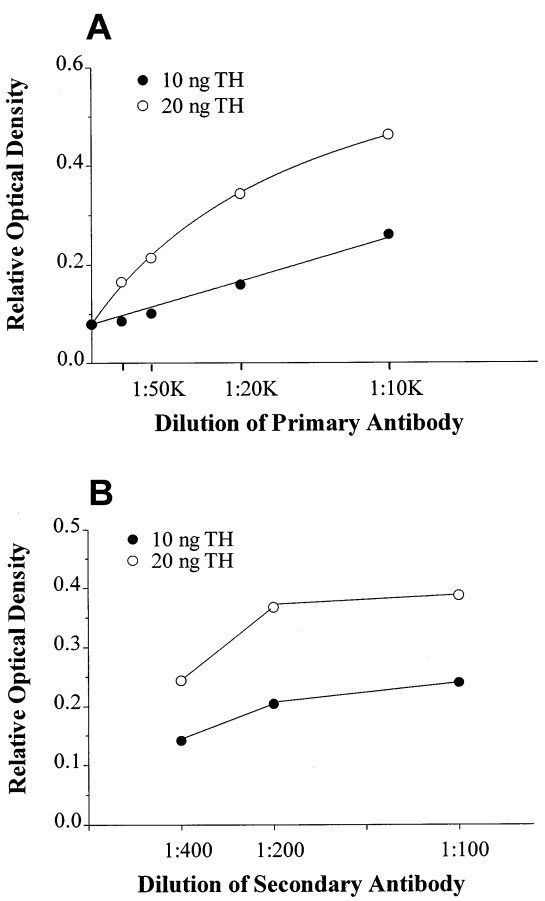

Fig. 1. The effect of primary antibody dilution on the relative optical density of recombinant TH dotted onto the membranes. The [^{125}I]secondary antibody dilution was 1:200. B. The effect of [^{125}I]secondary antibody dilution on relative optical density of recombinant TH dotted onto the membranes. The primary antibody dilution was 1:20 000.

3. Results

3.1. Determination of total protein parameters for TH standards

In order to establish a standard curve of TH for quantifying TH in tissue-transfers, experiments were performed to establish experimental parameters for TH standards. First, the capacity of protein binding to PVDF membranes was measured. A volume of 1 μl of protein preparation (homogenate of human cerebellum), spotted onto the membrane, occupied an area approximately equal to the compact cellular region of the human LC. Varying concentrations of protein, ranging from 0.1 to 3 μg in 1 μl were spotted onto PVDF membranes and dried. After staining with Ponceau S, optical densities of protein spots were measured with the aid of a computer. The amount of protein (<1 μg) spotted and dried onto the membrane was nearly linearly proportional to the relative optical density of the stained protein spot. Therefore, TH standards, containing varying concentrations of TH, were adjusted to a constant concentration of total protein (0.5 μg/μl) by adding the appropriate amounts of human cerebellar tissue homogenate.

3.2. Determination of antibody concentrations and generation of standard curve

Experiments were performed to determine the optimal concentrations of primary and secondary antibodies for tissue transfer-immunoblotting. Two concentrations of purified recombinant rat TH (10 and 20 ng) were used and total protein concentrations were adjusted to 0.5 μg/μl with human cerebellar protein, as described above. TH was spotted onto the PVDF membranes and various dilutions of primary anti-TH antibody and [^{125}I]secondary antibody were applied to determine the optical condition of the assay. First, using a fixed dilution of 1:200 of the [^{125}I]secondary antibody, the optical densities of the immunoblotted 10 and 20 ng TH standard spots were measured at varying dilutions of primary antibody (Fig. 1A). Second, the 1:20 000 dilution of primary antibody was selected to determine the effect of the varying dilution of the [^{125}I]secondary antibody on the optical density of the immunoblotted TH standards (Fig. 1B). All further experiments utilized a 1:20 000 dilution of primary anti-TH antibody and a 1:200 dilution of [^{125}I]labeled secondary antibody. Based on these results, a set of standards containing different amounts of purified recombinant rat TH were spotted onto PVDF mem-

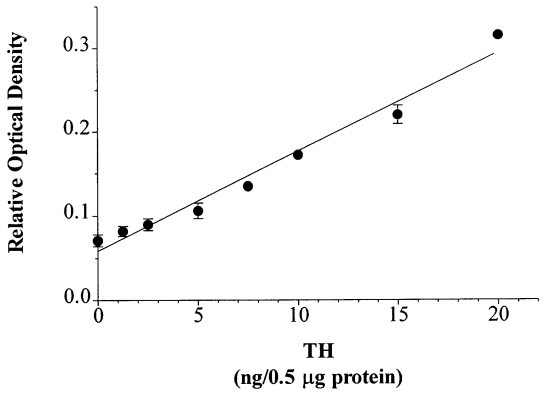

Fig. 2. The relationship between concentrations of TH standards and relative optical densities. Various concentrations of recombinant rat TH (in 1 μl) were dotted onto PVDF membrane and incubated sequentially with primary (1:20 000) and [^{125}I]secondary antibodies (1:200). All standards had equal total protein concentrations (0.5 μg/μl) adjusted with human cerebellum protein. Each point represents the mean value ± S.E.M. obtained from three separate experiments.

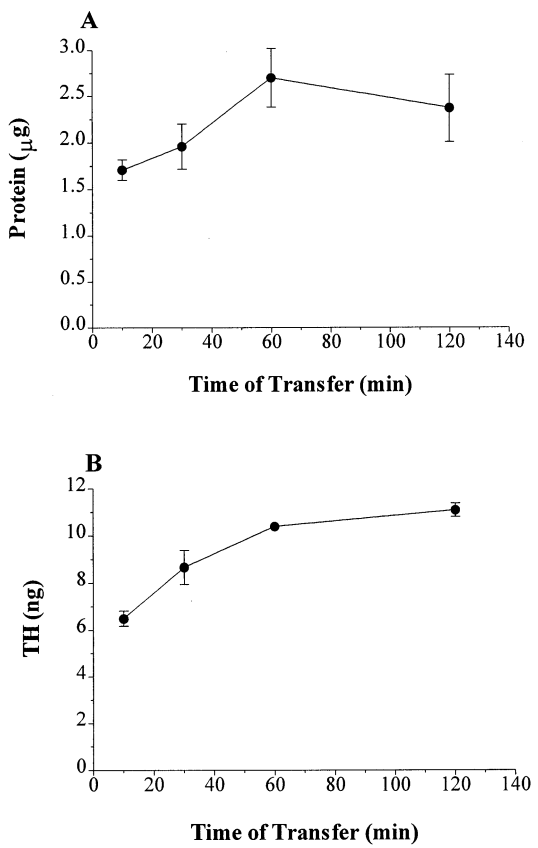

Fig. 3. The time course of the transfer of total protein (A) and immunoreactive TH (B) onto PVDF membranes. Protein was stained with Ponceau-S. Amounts of TH were determined by immunolabeling using a set of calibrated standards (as shown in Fig. 2). Each point represents the mean value ± S.E.M. obtained from three separate experiments.

branes. There was a significant correlation between relative optical densities of immuno-autoradiograms of TH standards and amounts of TH protein spotted onto the membrane ($r^2 = 0.98$, $P < 0.0001$, Fig. 2). Similar TH calibration curves were generated for each subsequent experiment.

3.3. Determination of transfer time

The optimal time for the transfer of TH protein from tissue sections was determined. First, transfers of sections of LC were performed successively onto PVDF membrane. That is, following a 1 h transfer, the same slide-mounted section was then placed on a fresh PVDF membrane for another hour, followed by a third hour of transfer on another fresh membrane. The amount of protein on the membranes was measured with the aid of a computer following Ponceau S staining. Approximately 93% of transferable proteins were transferred to the membrane during the first hour (data not shown). A second experiment was carried out to determine the maximum amount of proteins which can be transferred from the tissue section (15 μm). To do this, tissue remaining on the microscope slide following 1, 2 and 3 h transfers was scraped and the amounts of protein were determined. For each hour of transfer following the initial transfer, a fresh, wetted PVDF membrane was used. Other slides with tissue sections, which were cut adjacent and paired to those used for transfers, were only wetted with transfer buffer, dried and then scraped (controls, representing total protein prior to transfer). Amounts of proteins for tissue sections for the control tissue sections ($n = 4$ sections per group) were 259 ± 17 μg (paired with 1 h transfer), 278 ± 16 μg (paired with 2 h transfer) and 227 ± 12 μg (paired with 3 h transfer). The amounts of protein in sections remaining on the slide following 1, 2 and 3 h transfers were 203 ± 18, 219 ± 7 and 172 ± 6 μg ($n = 4$), respectively. Thus, the amount of protein transferred following 1 h, as a percentage of the total protein in the tissue section was 22%, i.e. $(259 - 203)/259 \times 100\%$. Likewise, the amount of protein transferred following 2 and 3 h transfers was 21 and 24%, respectively. This result verified that most of the protein that can be transferred to the membrane has been transferred to membrane within 1 h and that this transferable protein represents $\approx 22\%$ of the total amount of protein in the slide-mounted tissue section. Finally, quantitative protein (Ponceau S staining) and immunoreactive TH detections were carried out in a series of increasing transfer times from 10, 30, 60 and 120 min. Both stained protein and TH immunoreactivity were at nearly maximum levels following a 60-min transfer (Fig. 3). All transfers in the latter experiments utilized a 60-min transfer.

3.4. Effect of tissue thickness on transfer of TH and total protein

Experiments were performed to determine the effect of the thickness of tissue sections on the transfer of total protein to the PVDF membrane and on the amount of TH measured autoradiographically. Tissue sections containing human LC were cut at different thicknesses (5–40 μm) and transferred to membranes. The amount of protein bound to the membranes (determined by Ponceau S staining) was proportional to the section thickness over the range of section thicknesses studied. However, the amount of TH-immunoreactivity measured autoradiographically reached a maximum at section thicknesses exceeding 15 μm (Fig. 4). A tissue thickness of 15 μm was chosen for further experiments.

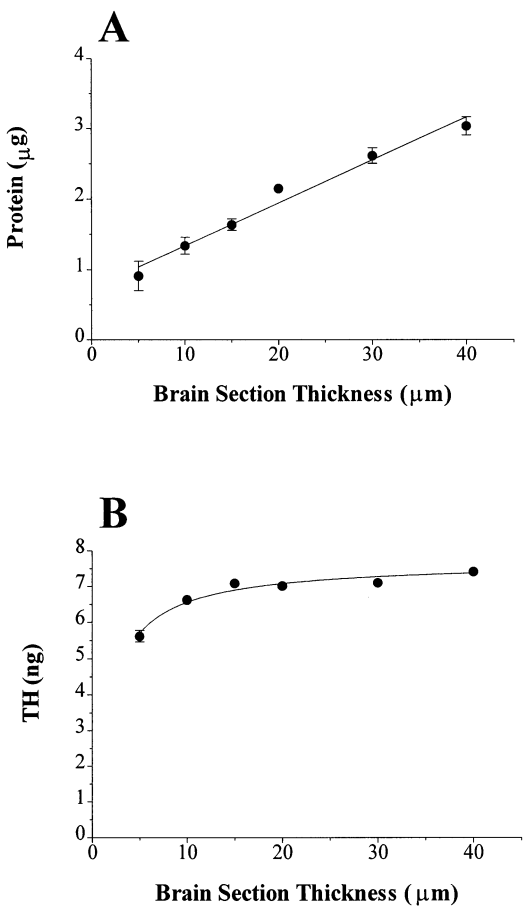

Fig. 4. The effect of thickness of tissue sections on the amount of (A) protein and (B) immunoreactive TH transferred to PVDF membranes following a 1-h transfer. Amounts of protein were determined following Ponceau S staining using a set of calibrated protein standards. Amounts of TH were determined using a set of calibrated standards as shown in Fig. 2. Each point represents the mean value ± S.E.M. obtained from three separate experiments.

3.5. Comparison of tissue transfer immunoblots with classic Western blotting

Experiments were performed to determine whether relative amounts of TH measured by the tissue transfer technique in the LC from different subjects were similar to the relative amounts of TH measured in the same samples using Western immunoblotting. To do this, protein from tissue sections containing LC from six human subjects were transferred to membranes and then processed using the procedures as described above. Densitometric measurements of autoradiograms were made using the Microcomputer Controlled Imaging Device (MCID M2). The LC was analyzed by simultaneously overlaying the image of the autoradiogram with the image of a histologically stained section cut adjacent to the section that was transferred. For the LC, the smallest region encompassing all cell bodies containing neuromelanin pigment was outlined. Other sections, cut adjacent to those used for tissue transfers, were used to measure TH by quantitative Western blotting. For these sections, the immediate region of the LC was punched (5 mm diameter bore) and processed for Western blots as described in Section 2. The amount of TH (in ng) measured in the LC from six human subjects was 3.38 and 0.61, 3.65 and 0.79, 4.00 and 0.80, 8.15 and 0.89, 13.18 and 0.94, 17.33 and 1.18 as determined using the tissue transfer technique and traditional Western blotting, respectively. Amounts of TH determined by the tissue transfer technique were correlated positively with amounts of TH-immunoreactivity determined by Western blotting (Table 1; $r^2 = 0.99$, $P < 0.01$).

3.6. Anatomical examination of TH along the axis of the LC

Using the method of tissue transfer immunoblotting, TH was measured at multiple levels along the rostro–caudal axis of the human LC. Images of autoradiograms of immunoreactive TH were overlaid with images of Cresyl violet-stained sections with the aid of a computer and TH was measured in the compact cellular region of the LC. The sections used for histological overlays were the same sections used for the tissue transfers because sufficient tissue remained on the slide to permit identification of LC cells. This overlay method permitted precise anatomical identification of the LC in the immunoreactive TH autoradiogram. Transfer autoradiograms demonstrate that TH is unevenly distributed along the rostro–caudal axis of LC in the human (Fig. 5). Furthermore, the amount of TH at each LC level was closely associated the number of neuromelanin-containing cells at each LC level (Fig. 6).

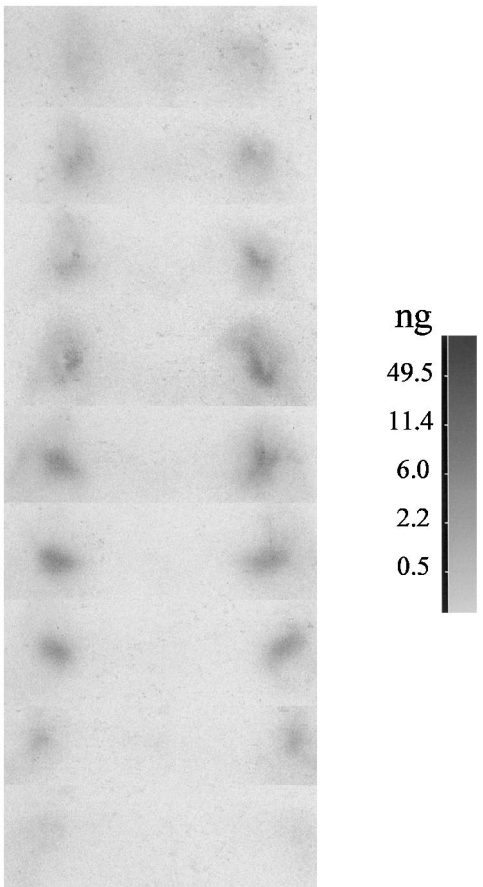

Fig. 5. Autoradiograms of TH at multiple transverse levels along the rostro–caudal axis of the human LC. Sections were cut at 1 mm intervals and numbers indicate the distance (mm) from the frenulum in the caudal direction. Concentrations of TH at levels 1 to 9 were 3.5, 5.8, 8.1, 10.7, 11.8, 9.1, 8.8, 5.1 and 3.2 ng, respectively (values are the average of both sides at each level).

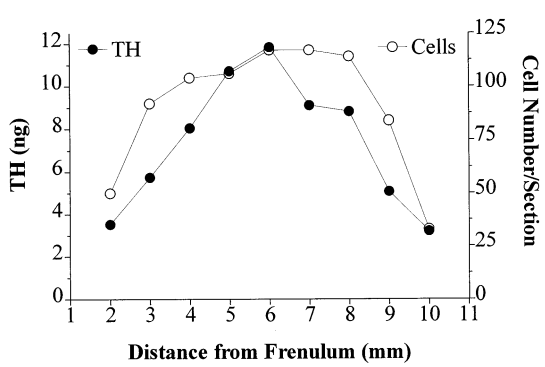

Fig. 6. Relationship between numbers of neuromelanin-containing cells and relative density of immunoreactive TH along the rostro–caudal extent of the LC (same subject as shown in Fig. 5). The abscissa is the distance from the frenulum in the caudal direction.

4. Discussion

The present study describes a method to measure immunoreactive TH in the LC of postmortem human brain following transfer of proteins directly from tissue sections to a hydrophobic PVDF membrane. PVDF membranes with bound proteins were subjected to successive incubations with an antibody for TH and a [^{125}I]labeled secondary antibody. Autoradiograms produced from this process revealed immunoreactive TH over the region of the LC with a moderate degree of anatomical resolution. The use of purified recombinant rat TH standard permitted more precise estimation of TH protein levels in the LC. Using this method, an uneven distribution of TH-immunoreactivity along the rostro–caudal axis of the human LC was demonstrated. Other investigators have utilized a similar method to transfer proteins from rat brain sections to nitrocellulose or other membrane matrices (Weissmann et al., 1989; Okabe et al., 1993). To our knowledge, the present study is the first demonstration of the use of a section transfer method to analyze human postmortem brain.

Several aspects of the transfer method were studied to optimize conditions for transfer of TH protein. It was assumed that not all protein in the tissue section would transfer to the membrane, regardless of the length of time of the transfer or of the thickness of the section, since it would be expected that some structural or membrane proteins would not be transferred. Our results showed that of total proteins that were transferable in the tissue section, most were transferred to the membrane in 1 h of transfer time from a 15-μm tissue section. Longer transfer times did not increase the amount of protein transferred (see Fig. 2A). Similarly, most transferable TH immunoreactivity was bound to the membrane in 1 h (Fig. 2B).

There was an apparent difference in the relationship between tissue section thickness and the amount of total proteins transferred and that between section thickness and TH protein transferred. The amount of total transferred proteins was proportional to the section thickness up to nearly 40 μm (largest thickness measured). In contrast, TH immunoreactive protein transferred did not vary linearly with section thickness and approached saturation at 15 μm of thickness. It should be noted here that two different methods to quantify protein were used: TH immunoreactivity was measured using immunoblotting ([^{125}I]secondary antibody), whereas total protein was measured using Ponceau S protein staining. Nevertheless, each method used a set of calibrated standards for comparisons and measurements were made well within the sensitive range of densitometry for the computer system utilized (MCID M2 System). While different measurement procedures may contribute to the above discrepancy, another possible explanation is that in a section thicker than 15 μm, very little TH can be transferred from the part of the tissue section that is >15 μm from the membrane (i.e.

there is a finite distance that TH is able to move in the tissue section). The fact that total protein transferred continues to increase at thickness >15 μm implies that membrane is not saturated with protein at a point when the maximum amount of transferable TH has moved to the membrane. From the standpoint of TH measurement, this is what one would prefer. That is, if there is more TH in the first 15 μm (e.g. in a disease state or following a drug treatment), then there is more available binding sites on the membrane to accommodate the additional TH protein.

Establishing a consistent relationship between the optical density of immunoreactive labeling on autoradiograms and the tissue concentration of the protein is a crucial part of any quantitative immunoautoradiographic method. For similar methods reported previously, calibration curves for TH quantitation were generated from Western blots (Weissmann et al., 1989) or TH antigen from tissue and standard calibration curves were assayed on different supports (fresh frozen tissue section vis a vis nitrocellulose, Raisman-Vozari et al., 1991). In the present paper, purified recombinant TH was used as a standard. To approximate conditions of TH in human tissue sections, the recombinant TH was mixed with human cerebellar homogenate to achieve constant total protein concentration (0.5 μg/μl) for each standard and was then dotted onto the same membrane to which the tissue section was transferred. This concentration and volume of protein was selected because: (1) it was in the range of concentrations that were not saturating the membrane; and (2) the area that 1 μl of the standard spreads onto the membrane produced a Ponceau S stain similar in optical density (magnitude) to the Ponceau S stain of the transferred 15 μm tissue section (not shown). Hence, proteins in standards and samples were bound to the same membrane in similar concentrations, and incubated simultaneously with primary and secondary antibodies, permitting contact of antibody to target molecules on the same support.

Quantitation of TH immunoreactivity in the tissue transfer procedure was validated by a parallel measurement of immunoreactive TH in LC by Western blotting of homogenates from the same subjects. The levels of TH in the LC measured using the tissue transfer method correlated highly with levels of TH measured using quantitative Western blotting. However, levels of TH measured by the transfer method were higher than levels measured by Western blotting. One would expect that the enhanced anatomical resolution in measuring TH would yield higher levels of TH. For example, circular punches with a 5-mm diameter (area of ≈ 20 mm^2) were cut from sections for Western blotting. In contrast, the immediate cellular region of the LC was measured in the tissue transfer method by overlaying images of adjacent sections that were histologically stained. This cellular region is not circular and has an area of ≈ 3–4 mm^2. Thus, in the transfer method, the discrete area of the LC can be identified and measured. In contrast, TH concentration is diluted by non-LC tissue in the large area around the LC that is punched in the Western blot method. However, even if a 5-mm diameter circle is used to measure TH in the autoradiograms of the tissue transfers, the amounts of TH exceed those measured by punch/Western blotting (data not shown). TH levels measured by autoradiographic analysis of films generated from tissue transfers are a function of the amount of TH protein and the area over which the TH protein is spread. In Western blotting, the area of the bands is held constant, at least theoretically, by using equal total protein loads. If TH standards for the transfers had consisted of the same amounts of TH protein, but diluted into smaller volumes that would spread over a smaller area, then the range of optical densities of the standards would have been higher. When optical densities of TH immunoreactive signal in the LC are compared to these standards, the estimated TH levels would be lower. Hence, amounts of TH estimated in the tissue transfer method are highly dependent upon the area over which the TH is spread. As such, this method yields pseudo-quantitative data that cannot be compared quantitatively to Western blotting data. Nevertheless, this tissue transfer method yields data that is more precise with respect to anatomical delineation of the LC in comparison to punch/Western blotting of the LC by avoiding variability and potential errors in dissection of this small structure.

One advantage of the present method, compared with the direct immunostaining of tissue sections, is that a standard curve can be generated by dotting known amounts of the antigenic protein onto the same membrane. The standard curve generated in this manner demonstrates a linear relationship between amounts of TH immunoreactivity and optical density of the film. This standard permits quantification of amounts of antigenic protein in test samples. Generation of a standard is difficult, if even possible, using methods involving direct immunostaining of tissue sections. Another advantage of the present method is that tissue fixation is avoided. Tissue fixation with paraformaldehyde or other compounds can potentially cause molecular changes in the antigens and can affect tissue penetration of large immunochemical markers (antibodies). A disadvantage of the transfer method as compared to direct immunostaining methods is that the cellular details of immunostaining are lost. However, cellular details are retained in the tissue section that remains on the slide following the transfer of protein and these were sufficient in the present study for Nissl staining and overlaying images (on a computer-aided image analysis system, see Section 2) of the section with the

immunostained membrane. The PVDF membranes used in the present method are less fragile than nitrocellulose and suitable for repeated washing and handling. If stored properly, these membranes with transferred proteins can be repeatedly blotted for immunoreactive detection of multiple proteins. Preliminary studies indicate that this method can also be applied to the measurement of TH in tissue sections of the striatum and hippocampus (data not shown). However, a possible limitation may be that not all proteins in tissue sections can be transferred to the membranes. Some proteins that are the structural or located in plasma membranes may be much more difficult to transfer (Okabe et al., 1993). For example, attempts by us to immunolabel the human norepinephrine transporter, a transmembrane protein, failed to generate a detectable signal over the LC region, while Western blotting the punched LC region did show a clear autoradiographic band.

In summary, tissue transfer immunolabeling combines the advantages of histochemical and immunochemical techniques for measurement of TH in a discrete brain region. This method will be a useful tool for exploring the modulation of TH expression in the human brain and the potential role of this enzyme in psychiatric diseases.

Acknowledgements

The authors gratefully acknowledge Dr Craig Stockmeier and Ginny Dilley (Case Western Reserve University) for helpful discussions and for assisting in the collection of brain tissue. The help of Dr Elizabeth Balraj and the staff of the Cuyahoga County Coroner's Office (Cleveland, OH) is also deeply appreciated. This research was supported by NIMH MH46692 and MH45488 and the American Foundation for Suicide Prevention.

References

Kostowski W, Plaznik A, Danysz W. The role of locus coeruleus-limbic noradrenergic transmission in the action of antidepressant drugs. Psychopharmacol Bull 1986;22:512–22.

Lake CR, Sternberg DE, VanKammen DP, Ballenger JC, Ziegler MG, Post RM, Kopin LJ, Bunney WE. Schizophrenia: elevated cerebrospinal fluid norepinephrine. Science 1980;207:331–3.

Levitt M, Spector S, Sjoerdsma A, Udenfriends S. Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. J Pharmacol Exp Ther 1965;148:1–8.

Melia KR, Duman RS. Involvement of corticotropin-releasing factor in chronic stress regulation of the brain noradrenergic system. Proc Natl Acad Sci USA 1991;88:8382–6.

Melia KR, Rasmussen K, Terwilliger RZ, Haycock JW, Nestler EJ, Duman RS. Coordinate regulation of the cyclic AMP system with firing rate and expression of tyrosine hydroxylase in the rat locus coeruleus: effects of chronic stress and drug treatments. J Neurochem 1992;58:494–502.

Nagatsu T, Levitt M, Udenfriend S. Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. J Biol Chem 1964;239:2910–7.

Nestler EJ, McMahon A, Sabban EL, Tallman JF, Duman RS. Chronic antidepressant administration decreases the expression of tyrosine hydroxylase in the rat locus coeruleus. Proc Natl Acad Sci USA 1990;87:7522–6.

Okabe M, Nyakas C, Buwalda B, Luiten PG. In situ blotting: a novel method for direct transfer of native proteins from sectioned tissue to blotting membrane: procedure and some applications. J Histochem Cytochem 1993;41:927–34.

Ordway GA, Smith KS, Haycock JW. Elevated tyrosine hydroxylase in the locus coeruleus of suicide victims. J Neurochem 1994;62:680–5.

Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977;83:346–56.

Prange AJ Jr. The pharmacology and biochemistry of depression. Dis Nerv Sys 1964;25:217–21.

Raisman-Vozari R, Hirsch E, Javoy-Agid F, Vassort C, Savasta M, Feuerstein C, Thibault J, Agid Y. Quantitative autoradiography of tyrosine hydroxylase immunoreactivity in the rat brain. J Neurochem 1991;57:1212–22.

Redmond DE, Huang YH. Current concepts. II. New evidence for a locus coeruleus-norepinephrine connection with anxiety. Life Sci 1979;25:2149–62.

Reis DJ, Joh TH, Ross RA, Pickel VM. Reserpine selectively increases tyrosine hydroxylase and dopamine b-hydroxylase protein in central noradrenergic neurons. Brain Res 1974;81:380–6.

Thoenen H. Induction of tyrosine hydroxylase in peripheral and central adrenergic neurones by cold exposure of rats. Nature 1970;28:861–2.

Thoenen H, Angeletti PU, Levi-Montalcini R, Kettler R. Selective induction by nerve growth factor of tyrosine hydroxylase and dopamine-β-hydroxylase in the rat superior cervical ganglia. Proc Natl Acad Sci USA 1971;68:1598–602.

Weissmann D, Labatut R, Richard F, Rousset C, Pujol J. Direct transfer into nitrocellulose and quantitative radioautographic anatomical determination of brain tyrosine hydroxylase protein concentration. J Neurochem 1989;53:793–9.

Zigmond RE. Tyrosine hydroxylase activity in noradrenergic neurons of the locus ceruleus after reserpine administration: sequential increase in cell bodies and terminals. J Neurochem 1979;32:23–9.

Zigmond RE, Schwarzschild MA, Rittenhouse AR. Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. Ann Rev Neurosci 1989;12:415–61.