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Original Article

Immunocytochemical Detection of Regional Protein Changes in Rat Brain Sections Using Computer-assisted Image Analysis[1]

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We used several approaches to assess the reliability and sensitivity of computer-assisted densitometry to detect regional changes in tissue antigen content as a function of immunohistochemical staining density. We designed a model system to mimic variations in antigen concentration in postfixed, slide-mounted rat brain sections by varying the ratios of conjugated (biotinylated) to unconjugated secondary antibody. Antigen concentration was also varied in tissue discs made from mixing rat brain homogenate with increasing amounts of tissue embedding compound. The monoclonal antibody bd-17 to the $\beta_{2/3}$ subunit of the $GABA_A$ receptor was used as the primary antibody. Immunostaining density was visualized with diaminobenzidine (DAB). There was a significant, positive linear relationship (r = 0.97–0.99) between immunostaining intensity and antigen concentration. With this approach, changes in antigen content of less than 10%, as reflected in immunostaining intensity, were detectable in brain sections. The low degree of variability in measures of regional variation in immunostaining in sections from naive rats (n = 7) suggested that the method was suitable for quantitative analysis and indicated the reliability of the method. This systematic study of the utility of computer-assisted image analysis for semiquantitative immunohistochemical analysis found the method to be both reliable and sensitive. (*J Histochem Cytochem 44:981–987, 1996*)

KEY WORDS: Immunohistochemistry; Image analysis; $GABA_A$ receptor; Rat; Brain.

Introduction

Immunocytochemistry is a widely used general scientific method and neurohistochemical technique (Farr and Nakane, 1981). There are many situations in which the amount of antigen may change in a particular brain region, e.g., as the result of an experimental treatment, as a function of development, or in neuropathological conditions. Moreover, the detection of receptor proteins is at present an intense area of interest because mRNA changes in brain detected by in situ hybridization methods do not necessarily reflect changes in the levels of translated protein. Nevertheless, quantitative analysis using immunocytochemical techniques is not widely accepted. A main concern is the reliability of measurements owing to the variations in staining density that may result from inconsistent tissue fixation and subsequent immunocytochemical procedures (Larsson, 1988a,b), particularly when comparisons of immunocytochemical staining density are made among a number of samples within or across experimental groups.

Subjective judgments of immunostaining density have typically been used to compare the relative abundance of an antigen in different regions of the same tissue section in which fixation and immunocytochemical methods are presumed constant. It is further assumed that relative immunostaining densities are reasonably proportional to the antigen content in the various brain regions. Subjective estimates of relative antigen concentration can be extended only with caution to other tissue sections from the same animal or to those from other members of the group. A number of investigators have more recently used immunostaining densities to make semiquantitative estimates of protein or antigen concentration (Gutierrez et al., 1994; Livingston et al., 1993; Woodson et al., 1989). However, the reliability and accuracy of these methods for quantitative analysis of antigens in brain tissue have not been systematically studied and there is no evidence for the precision of such estimates. Furthermore, no studies have addressed the reliability of between-group estimates or, more importantly, whether such measures reasonably and accurately reflect the relative amount of antigen in brain tissue.

Computer-assisted image analysis can greatly facilitate accurate measurement of regional variations in optical density (Altar et al., 1984; Porro et al., 1984). With such systems, relative gray values, reflecting optical density, can be used to gauge immunostaining intensity on tissue sections. Comparison of relative gray values pro-

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vides a more objective and reliable method than the aforementioned arbitrary judgments typically used to estimate relative abundance of receptor proteins or other cell components of interest.

This study was designed to systematically evaluate the effect of both simulated and actual variations in antigen content on immunostaining intensity and to determine the usefulness of computer-assisted image analysis to reliably detect relative variations in immunostaining density in postfixed thin sections of rat brain. Two approaches were used to simulate variations in antigen content. First, the ratios of unconjugated to conjugated (biotinylated) secondary antibody (Ab) were varied in rat brain sections to mimic changes in the density of immunostaining reflecting the primary Ab interaction with the antigen. Second, antigen concentration was varied in rat brain homogenate diluted with increasing concentrations of an inert tissue embedding compound. Tissue discs cut from serially diluted brain homogenate were then subjected to standard immunocytochemical procedures. Finally, normal regional variations in antigen content were assessed in brain sections derived from a group (n = 7) of naive rats.

Materials and Methods

Tissue Preparation. Adult male Sprague–Dawley rats (Harlan; Haslett, MI) were used to obtain frozen brain sections and to provide tissue for brain homogenate discs. The institution's policies, which adhere to the National Research Council's guide for the care and use of laboratory animals, were followed. Rats were anesthetized with ketamine (80 mg/kg IM) and transcardially perfused for 1 min with ice-cold saline (0.9%) at a constant rate of 150 ml/min. Rat brains were dissected and frozen in isopentane in an acetone–dry ice bath. After a 1-hr equilibration, brains were sectioned (10 μm) with a cryostat in the parasagittal plane and thaw-mounted on poly-L-lysine-coated slides. The cryostat knife setting was kept constant over the entire sectioning process for both brain sections and tissue discs.

For tissue homogenate discs, whole brains, minus the brainstem, were homogenized in a Potter–Elvejheim tube with a Teflon pestle attached to a high-speed drill. Brain tissue homogenate was mixed with tissue embedding compound (OCT; Miles Scientific, Elkhart, IN) in serial ratios from 20 to 100% (w/w) of OCT compound to brain homogenate. Brain homogenate with different ratios of OCT were centrifuged (1000 × g for 1 min, 4°C) in polyethylene cylinders and frozen in isopentane in an acetone–dry ice bath. Brain homogenate columns cut from the polyethylene tubes were sectioned into 10-μm discs and thaw-mounted onto poly-L-lysine coated slides. Protein content was measured by the method of Lowry et al. (1951).

Immunohistochemistry. Brain sections and brain paste discs, brought to 24°C under vacuum, were postfixed with 4% paraformaldehyde plus 0.2% picric acid for 8 min, followed by two 5-min washes in 1 × PBS, pH 7.4. Fixed sections were blocked for 30 min at 24°C in 20% normal goat serum (v/v) in PBS plus 0.2% Triton X-100 (v/v). Sections were incubated overnight at 4°C with the primary Ab, bd-17 (20 μg/ml; Boehringer Mannheim; Indianapolis, IN). This monoclonal antibody (MAb) was generated by immunizing Balb/c mice with a highly purified $GABA_A$–benzodiazepine receptor from bovine cerebral cortex. MAb bd-17 recognizes the β-subunit of the rat $GABA_A$ receptor (Schoch et al., 1985). After three 5-min washes in PBS, sections were incubated with the secondary biotinylated Ab (1:250 dilution, anti-mouse IgG; Boehringer Mannheim) for 1 hr at 24°C. Sections were washed three times for 5 min in PBS, then incubated with avidin–biotin–peroxidase complex (ABC; Vector, Burlingame, CA) for 1 hr. Specific binding was visualized with 0.07% diaminobenzidine (DAB; Sigma, St Louis, MO), 0.02% hydrogen peroxide in PBS. Sections were dehydrated through a series of alcohols (75%, 95%, and 100%), cleared in xylene, and coverslipped with Permount (Fisher Scientific; Fair Lawn, NJ). Immunocytochemical procedures for immunostaining of brain homogenate discs were identical to those for brain sections.

To mimic the changes in staining intensity associated with antigen variation in brain sections, five consecutive sections were incubated with the same concentration of the primary Ab. The sections were then incubated with increasing ratios of unconjugated to biotinylated secondary Ab (0:100–80:20) for 1 hr and visualized with DAB. This procedure was repeated on brain sections from four rats. To maintain acceptable timing across sections, it was necessary to carry out this experiment by applying the five concentrations of secondary Ab on sections derived from each rat on 1 day, over a total of 4 days.

Another experiment, which more closely mimicked a typical experimental protocol, was designed to evaluate the sensitivity of image analysis as a semiquantitative measure to detect differences in immunostaining intensity. Gray values over images of immunocytochemically stained brain sections from naive rats (n = 6) were compared after incubation with biotinylated and unconjugated secondary Ab applied in ratios of 100:0 and 90:10 as described above and were visualized with DAB.

Finally, to assess normal variations in antigen content, brain sections from seven naive rats were immunostained with MAb bd-17 (20 μg/ml) and the biotinylated secondary Ab using the same immunocytochemical procedures described above.

Data Analysis. The immunostaining intensities over discrete brain regions and over brain homogenate discs of different protein contents were acquired and analyzed with a Macintosh-based computer-assisted image analysis system with the aid of NIH Image Software (v. 1.58). Slide-mounted tissues were placed on a light box (Northern Light; Imaging Research; St. Catherines, Ontario, Canada) which provided constant illumination from underneath the slide. Light box intensity was set to optimize the visibility of and the differentiation among different brain subregions on the tissue section. Images of entire tissue sections or areas of interest were acquired with a high-resolution CCD camera (Sierra Scientific; Sunnyvale, CA), with aperture and focus settings similarly adjusted to provide an optimal image. The overall illumination was also adjusted so that the distribution of relative gray values, i.e., number of pixels in the image as a function of gray value (0–256), fell within the limits of the system, typically within 30–220 gray value units, avoiding a floor or ceiling effect. Once established, the settings remained constant for all images acquired within a particular experimental protocol. Therefore, when all parameters were fixed, only tissue staining intensity influenced the measured gray value. Images, acquired as described, were digitized and stored on an external Bernoulli disk (Iomega; Roy, UT) for later analysis with a Macintosh Quadra 950 computer.

Gray value (per mm^2) measurements, which reflected immunostaining intensity, were made on digitized images by delimiting an area of interest, free-hand, using predetermined criteria to define the region or subregion. For experiments on brain sections, background gray value determined over the corpus callosum, a white matter area, was subtracted from the gray value determined over gray matter areas of brain. Because a comparable area could not be defined in brain homogenates, background gray values were not determined on tissue discs.

All data were expressed as mean ± SEM of the raw gray level values. Correlation coefficients derived from linear regression analyses were analyzed by t-test. A comparison of regional bd-17 staining patterns in naive rat brains was made by one-way analysis of variance. Individual comparisons were made with orthogonal contrasts.

Results

Analyses of simulated variations in antigen content in rat brain sections and in tissue discs yielded similar results. The five consecu-

tive brain sections incubated with increasing ratios of unconjugated and biotinylated secondary Ab showed a significant ($p < 0.02$) positive linear relationship between immunostaining intensity (relative gray value) and the concentration of biotinylated secondary Ab, each concentration representing the corresponding percentage antigen reduction (Figures 1 and 2). The coefficient of regression of immunostaining density vs the concentration of biotinylated secondary Ab was 0.99 in two of four experiments and 0.98, 0.97 in the remaining two.

The mean immunostaining intensity in the frontal cortex was significantly ($p < 0.001$) lower in brain sections incubated with a 90:10 ratio compared to 100:0 (Figure 3). A significant difference in immunostaining density was also detected in preliminary experiments in which conjugated to unconjugated secondary Ab ratios of 100:0 and 80:20 were used (data not shown). Therefore, on the basis of the former experiment, the sensitivity of this method was estimated to be able to detect antigen content changes as small or less than 10%.

There was a significant linear relationship between the proportional decrease in antigen concentration in brain homogenate discs and immunostaining density ($r = 0.98$; $p < 0.05$). Immunostaining density decreased as an increasing proportion of OCT compound was mixed with brain homogenate, i.e., with decreasing protein concentration (Figure 4D).

The distribution of bd-17 immunostaining across several brain regions of naive rats ($n = 7$) is illustrated in Figures 1 and 5. The relative density of immunoreactivity in parasagittal sections was similar to that of Schoch et al. (1985) and Gutierrez et al. (1994). As

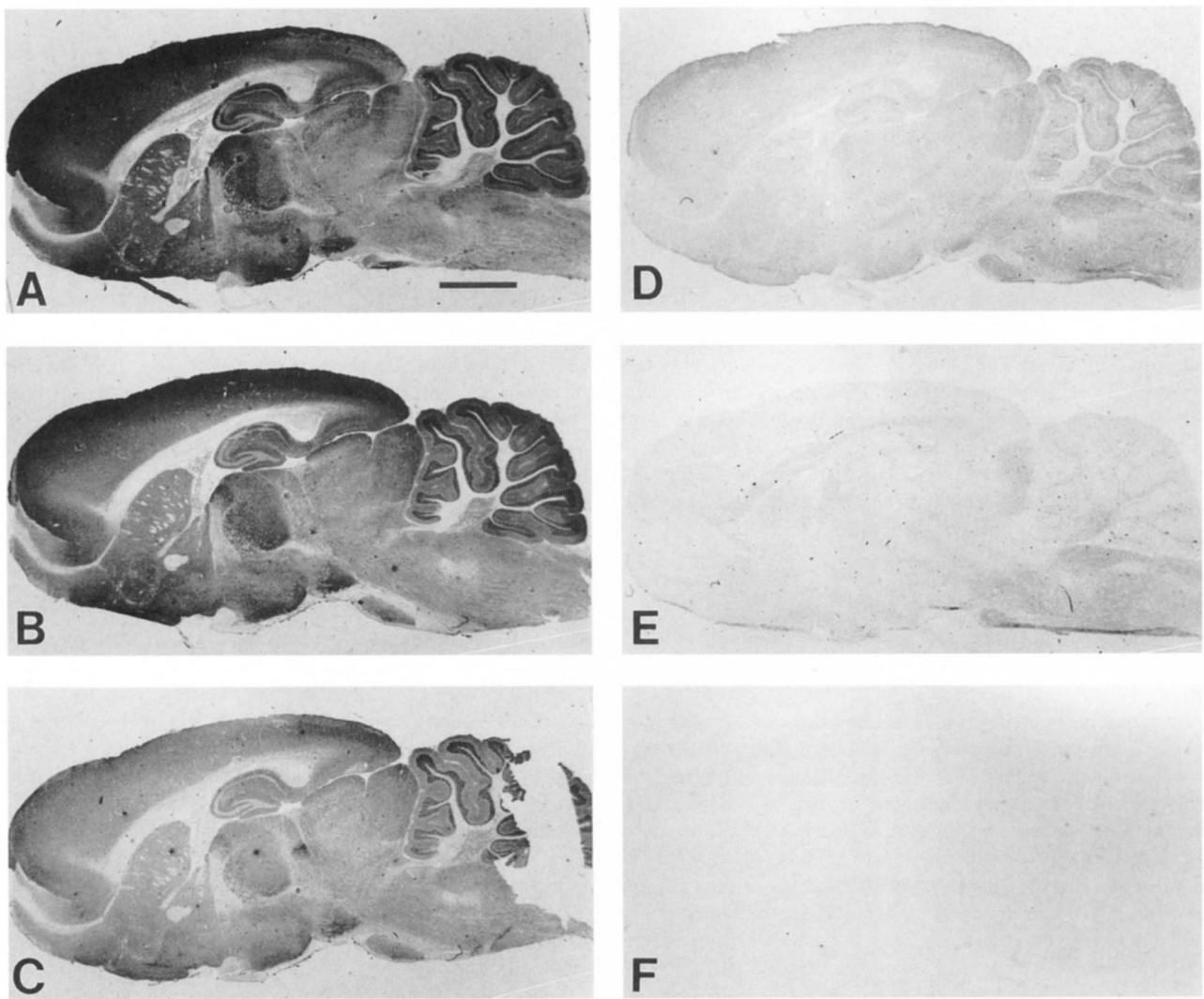

Figure 1. Immunostaining of brain sections with MAb bd-17, which crossreacts with the β_2- and β_3-subunits of the $GABA_A$ receptor. Five consecutive brain sections from a naive rat were incubated with the primary Ab, followed by incubation with biotinylated and unconjugated secondary Abs in the ratios indicated. **(A)** 100%; **(B)** 80%; **(C)** 60%; **(D)** 40%; **(E)** 20%; **(F)** negative control. The staining density of brain sections varied in parallel with the biotinylated secondary Ab concentration, which closely mimicked the antigen concentration reductions in brain tissues. Density measurements are shown in Figure 2. In the negative control **(F)** no primary Ab was applied. Bar = 2.5 mm.

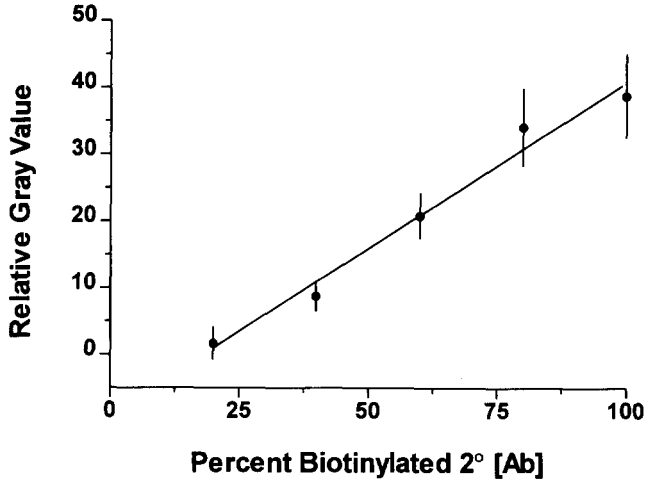

Figure 2. Relationship between immunostaining density and antigen concentration. Biotinylated secondary Ab was diluted with increasing amounts of unconjugated secondary Ab to mimic the effect of decreasing antigen concentration in brain sections. Dilutions of the biotinylated secondary Ab were applied to five brain sections (Figure 1) from each of four naive rats. Measurements are from a representative experiment. There was a strong positive correlation (r = 0.97–0.99; $p < 0.02$; n = 4) between relative immunostaining densities and increasing ratio of biotinylated secondary Ab. All measurements were from the frontal cortex.

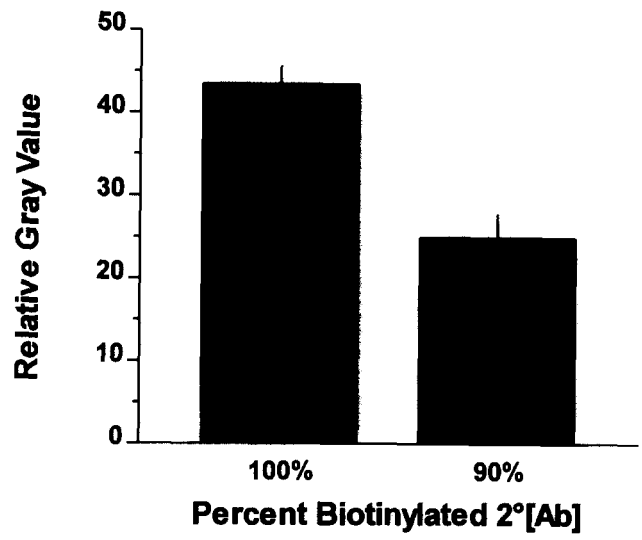

Figure 3. Sensitivity and reliability of computer-assisted measurements. Adjacent brain sections from six naive rats were incubated with biotinylated secondary antibody (2°[AB]) diluted with unconjugated secondary Ab as described in Figures 1 and 2. The relative staining density (mean ± SEM) measured with a conjugated secondary Ab concentration of 100% represented the basal antigen concentration. With a 90% secondary Ab concentration, staining density mirrored a 10% significant ($p < 0.0001$) decrease in antigen concentration and reflected the sensitivity of computer-assisted measurements.

shown in Figure 5, there were significant ($p < 0.0001$) differences in staining density among brain areas. For example, of those areas measured, the caudate had a significantly lower immunostaining density vs all other brain areas except for the thalamus. The thalamus had a lower staining density than the occipital–parietal cortex and the hippocampus. Immunostaining density was also different among cortical areas. The relatively small variation in immunostaining density across rats was indicated by the relatively small SEM.

Discussion

This study systematically investigated the accuracy and usefulness of computer-assisted image analysis of immunostaining density as a quantitative method to detect regional changes in the amount of a specific protein or other antigen of interest. Two approaches were used. First, a model system was designed to mimic variations in the density of immunostaining associated with a reduction in antigen concentration by varying the ratios of conjugated (biotinylated) to unconjugated secondary antibody in postfixed, slide-mounted thin sections. Antigen concentration, presumed to be proportional to protein content, was also varied in tissue discs made from rat brain homogenate in combination with increasing amounts of tissue embedding compound. The results indicated that immunostaining density in brain sections and tissue homogenates reflected the relative amount of antigen with reasonable accuracy.

The development of a model system for quantitative immunocytochemistry had previously been attempted by Moriarty et al. (1973) and Petrali et al. (1974). Both studies found that immunostaining intensities in rat brain sections were decreased with increasing dilutions of antiserum. Although these studies were originally designed to compare the sensitivity of immunocytochemistry to radioimmunoassay, these findings suggested, as pointed out by Leblond (1979), that the intensity of the DAB reaction is proportional to the quantity of antibody found by the section. These results also implied that the DAB reaction and densitometric measurement were potentially useful for quantitative measurement of antigens in immunocytochemical studies. Compared to these model systems, the model system in the present study was more predictable and more closely mimicked true immunocytochemical experimental conditions in which the concentration of the primary antibody is usually in excess relative to the concentration of antigen under study. Furthermore, the use of computer-assisted image analysis to measure the immunostaining density of discrete brain regions facilitated measurements and is more accurate and more objective. Therefore, both our results and theirs point to the fact that densitometric measurement of immunocytochemical staining by the enzymatic color reaction, particularly the DAB reaction, can be used as a quantitative method of detecting antigen content in brain sections. Moreover, computer-assisted image analysis may also have broader applicability to tissue types that may not be entirely uniform, as imaging software has the flexibility to allow addition or subtraction of regions or subregions of interest.

Our model system was based on the following facts or assumptions: (a) the bd-17 primary antibody is specific for its corresponding antigen, i.e., the $\beta_{2/3}$-subunit of the $GABA_A$ receptor and the working concentration of bd-17 was in excess of the antigen concentration; (b) the secondary antibodies, biotinylated and unconjugated, were specific for mouse IgG, the same class as bd-17; (c) the antigen concentrations in the brain regions of interest were constant across adjacent brain sections from the same rat; and (d) the antibody–antigen interactions were the same for the biotinylated and unconjugated secondary antibodies used.

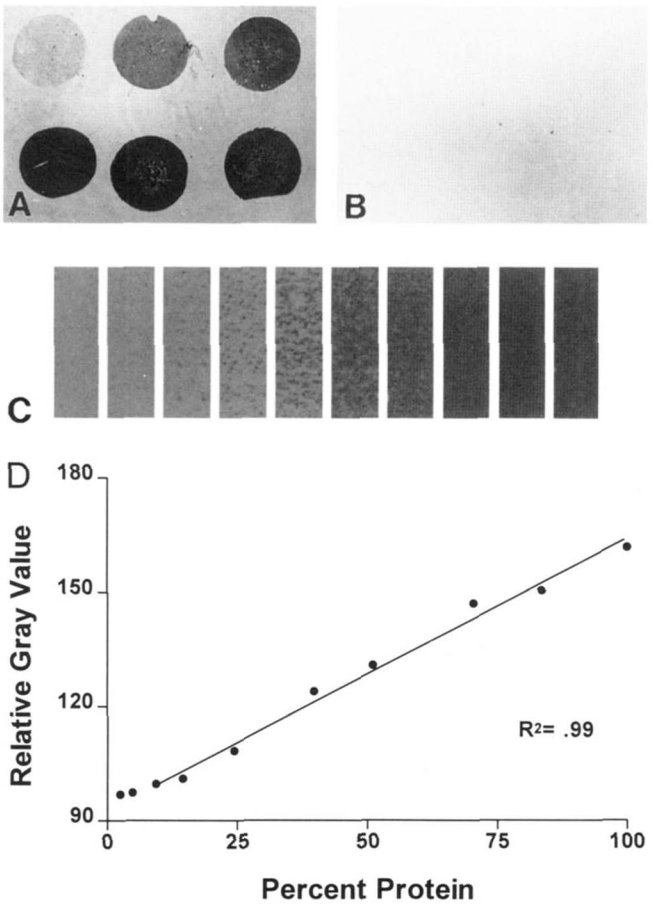

Figure 4. Simulated variation in antigen concentration in tissue discs. (**A**) Photomicrograph of brain paste discs containing increasing amounts of protein (25–100%) in which MAb bd-17 and the DAB reaction were used to detect the antigen. Viewed in a clockwise direction, protein concentration in the discs increases (25, 45, 55, 75, 85, 100%). (**B**) Negative control for the antibody reaction shown in **A**. (B) The primary Ab was omitted from the immunostaining procedure. (**C**) Magnified image of tissue discs illustrating antigen density with increasing protein concentration varying from 2.5 to 100%. Note the homogeneous distribution of antigen throughout the image. (**D**) Immunostaining of brain paste discs with antibodies to $GABA_A$ receptor subunits was measured in each of 10 tissue discs (n = 2–5 sets) using computer-assisted image analysis. Brain homogenate discs were reacted with the primary Ab bd-17. Immunostaining density increased linearly with increasing protein concentration, proportional to the antigen content in the discs. Data points representing protein contents <10% were analyzed using a least-squares best fit of the data to a linear function (r = 0.99; p <0.05; n = 3).

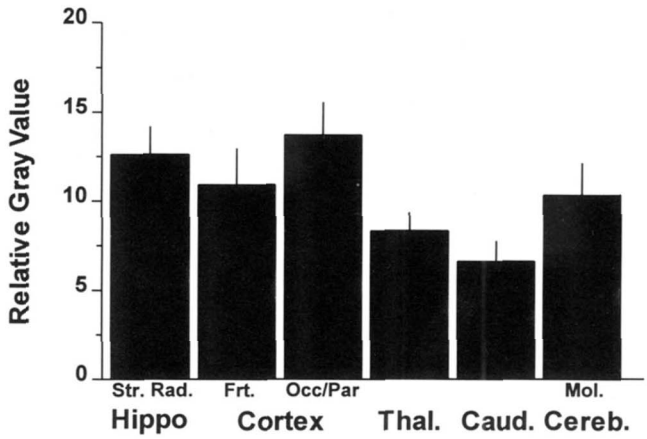

Figure 5. Reliability of computer-assisted measurements of immunostaining. Brain sections from six naive rats were reacted with MAb bd-17, which cross-reacts with the β_2- and β_3-subunits of the $GABA_A$ receptor. There were relatively small variations within the same brain region across different brain sections, reflected in the small SEM. Moreover, there were significant variations in staining density between brain regions within brain sections.

The specificity of bd-17 is not in question (Haring et al., 1985; Schoch et al., 1985). The results of negative control trials indicated the high specificity of the secondary antibody used. The significant positive linear relationship between immunostaining density and antigen concentration (Figure 2) supports the third and fourth assumptions. Finally, the observation that biotin-labeled and unlabeled antibodies have similar antibody–antigen interactions (Guesdon et al., 1979) justifies our use of mixed conjugated and unconjugated secondary antibodies in our immunocytochemical model.

The avidin–biotin–peroxidase complex (ABC) detection method has been found to be the most sensitive among several other detection methods, such as the standard peroxidase–anti-peroxidase (PAP) method and indirect immunoperoxidase procedures (Larsson, 1988b; Hsu et al., 1981). Moreover, diaminobenzidine (DAB) appears to be more sensitive than other chromogens (Larsson, 1988b; Trojanowski et al., 1983). In the early stages of the development of the present model, the PAP method and the nonamplified indirect immunoperoxidase procedure (data not shown), as well as ABC methods, were tried with bd-17 as the primary antibody. Similar to the findings of Larsson (1988b) and Hsu et al. (1981), our results showed that the ABC method provided the strongest signal. Compared to other methods, the ABC method involves extra steps that may influence the accuracy of the procedure or add more error variance to the quantitation, as suggested by Leblond (1979). In spite of these extra steps, the ABC method worked very well in reflecting the relative levels of antigen in our immunocytochemical model system, as demonstrated in Figures 1 and 2. The regression coefficient averaged 0.98 in studies in brain sections (Figure 2) as well as in the tissue disc experiment (Figure 4).

The experiments in tissue discs (Figure 4) derived from brain homogenates also demonstrated the usefulness of the latter model as a preliminary test of whether the immunocytochemical methods derived for a particular primary antibody and tissue type will yield linear, and therefore quantifiable, results. This method was intended to be used to model the effects of changes in protein concentration reflecting changes in antigen concentration in tissues and thus immunostaining intensity. It was not intended to be used as a standard to reflect the antigen–antibody reaction in intact tissues, which may be different from those in tissue homogenates. Therefore, although the tissue discs derived from brain homogenates are similar to those typically used as receptor autoradiographic standards (Tietz et al., 1986), this does not imply that they should be used in a similar manner in immunocytochemical experiments, e.g., for comparison with immunostaining density over brain sections. The relative gray values obtained over tissue homogenates would not be expected to be comparable to those over intact tissue sections because optimal image acquisition settings would be expected to

vary between protocols and tissue types. For example, differences between antibody staining in tissue discs and brain slices may vary because of differences in tissue fixation, antibody penetration, or the kinetics of the reaction. Therefore, in the present experiment direct comparisons were not made between gray levels (minimal and maximal values and the slope of the gray level–staining intensity relationship) among different protocols.

The sensitivity of immunocytochemical methods for detecting changes in antigen concentration was estimated by staining brain sections from naive rats with biotinylated vs unconjugated secondary antibody. Some of the inherent variabilities associated with immunocytochemical procedures were overcome by use of careful tissue handling techniques, e.g., postfixation and careful timing of procedural steps. Then it was determined whether a linear relationship could be established between relative gray level, which reflects immunostaining intensity, and conjugated–unconjugated secondary antibody concentration (Figures 1 and 2). Despite the usefulness of this measure, these experiments with multiple concentrations of secondary antibody had to be carried out across a number of days and therefore did not represent normal experimental conditions. Therefore, this experiment was repeated on one day in sections from naive rats, a more typical experimental protocol (Figure 3). The results suggested that changes in antigen content as small as or less than 10% could be detected. The reliability of the method was also demonstrated, i.e., the result of measurements of regional immunostaining in naive rat brain sections (Figure 5), as would be routine in many experiments, showed relatively small variations in staining density within the same brain area across different rat brain sections, whereas significantly different staining densities existed in different brain regions in the same brain sections. The relatively high sensitivity and reliability of the method further support its suitability for quantitative analysis.

For estimating potential changes in antigen concentration across experimental groups, a positive control, i.e., an antigen that is presumably invariant, is desirable to underscore the specificity of the change in the antigen of interest. Cellular structural element proteins appear to be good candidates for this purpose, because they are presumably more stable than other cellular proteins. Several antibodies against cellular structural proteins are commercially available, such as antibodies against β-actin, neurofilament protein, and glial fibrillary protein (GFAP). Immunostaining with antibodies against neurofilament protein and GFAP on thin (10-μm) brain sections was tested in the present study (data not shown).

However, unlike the results of Gutierrez et al. (1994), the staining for these proteins on the brain sections was not homogeneous. Instead, only discrete cell somata and large dendrites showed immunoreactivity. Furthermore, the number of GFAP-stained cells varied considerably between adjacent brain sections, with immunostaining density varying by as much as twofold in terms of gray values measured by computer densitometry. This discrepancy may be due to the thin sections (10 μm) used in the present study compared to the 25-μm sections used by Gutierrez et al. (1994). The antibody against neurofilament protein also stained only individual cells. The results indicated that these two cellular structural proteins were not good candidates as positive controls in thin sections. The alternative may be the use of antibodies to other cellular proteins or the use of antibodies for receptor proteins of the same or other neurotransmitter systems which, according to other supportive experimental evidence, would not be expected to change. Indeed, preliminary evidence from our laboratory using these techniques (Huang et al., 1995) indicated a regional decrease in several brain areas in the relative amount of protein associated with the α_1- and β_3-subunits of the $GABA_A$ receptor after 1 week of benzodiazepine treatment, whereas the staining for the $\beta_{2/3}$-subunit was not changed. Therefore, antibodies that reacted with the same $GABA_A$ receptor complex served as excellent positive controls.

Finally, in the present study we found that several steps in the immunostaining procedure were important in minimizing variations in immunostaining, thus enhancing the sensitivity and reliability of these methods. For example, the cryostat knife setting was controlled so that only normal systematic error due to section thickness contributed to staining intensity. With respect to tissue fixation, all rats were perfused with saline; only then were sections postfixed together for the same length of time for each experiment. Consequently, the effects of the fixative on the antigens in the brain section were identical to or equivalent for each individual section. In addition, no part of the brain section was allowed to dry out between any steps in the process. Each working solution was applied when sections were still wet after the previous washing. In addition, all immunostaining steps were carried out in slide-mounted rather than in floating sections, which greatly facilitated the control of the time for each step, especially the time for the final DAB–peroxidase reaction.

Limitations were also noted with this method. For example, not all primary antibodies are good candidates for use on brain sections thaw-mounted on slides. Likewise, some primary antibodies do not work well in postfixed brain sections. These primary antibodies require prefixation and floating procedures, which could potentially induce variations in immunostaining density. Caution should also be exercised in using this approach if the antigen under study is considerably smaller than the primary antibody molecule. As pointed out by Larsson (1988c), this potential problem would be magnified in situations in which the antigen concentration at a particular site is high, resulting in an underestimation of antigen concentration. The excellent positive linear relationship between immunostaining density and the concentration of protein, i.e., presumably antigen in brain homogenate discs, did not suggest this possibility.

In conclusion, analysis of immunocytochemical staining with computer-assisted image analysis, can be a reliable and sensitive method for determining the relative levels of antigen in tissue sections to detect discrete regional changes in specific proteins of interest and is likely to have applicability to a variety of other tissue types.

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