

Chapter 12

Analysis of the Expression Profile and Regional Distribution of Neurotransmitter Receptors and Ion Channels in the Central Nervous System Using Histoblots

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Abstract

The histoblot method is a reliable and convenient way to compare the regional distribution and expression level of different proteins in brain samples without compromising the integrity of antibody-binding sites by tissue fixation, which is required for conventional immunocytochemistry. Fixation introduces covalent modifications, cross-linking and/or denaturation of proteins. These chemical modifications often alter the antibody-binding sites, and cross-linked molecules may hinder the access of antibody to epitopes. The direct transfer of native proteins from unfixed frozen tissue sections to an immobilising matrix offers much improved accessibility of the transferred proteins for immunochemical analysis. The histoblot method has been successfully applied to analyse the regional distribution of several neurotransmitter receptors, ion channels and other proteins in the adult and developing brains. While this technique lacks cellular resolution, it provides high sensitivity and much improved consistency compared to conventional immunohistochemical techniques, which is essential for reliable quantitative comparisons of overall expression levels of proteins in different brain regions. Compared to conventional immunoblot analysis of protein extracts from dissected brain regions, histoblots provide more accurate and direct information about the anatomical localisation of proteins. In this chapter we describe the histoblot protocol we have used for the identification of quantitative changes in a wide range of neurotransmitter receptors and ion channels in various brain regions.

Key words Alkaline phosphatase, Antibodies, Cryostat section, Expression level, Histoblot, Immunoblot, Immunostaining, Localisation, Nitrocellulose membrane

1 Introduction

Mapping of the regional expression profiles of various receptors, ion channels and other proteins is frequently applied for a wide range of studies of the central nervous system (CNS). While conventional immunohistochemical techniques are often able to reveal the anatomical distribution of endogenous proteins in the brain tissue at relatively high resolution, they also have several limitations that could undermine protein target recognition by antibodies [1].

Immunohistochemical analysis requires the fixation of the brain tissue to preserve morphology and to protect samples from the rigours of processing and staining techniques. Fixatives stabilise cells and tissues via either introducing covalent cross-linkages (e.g. aldehydes such as glutaraldehyde or formalin) or by denaturing proteins (e.g. acetone and methanol) or a combination of both. These changes in target proteins often reduce the ability of antibodies to access and/or recognise their epitopes in fixative-treated tissue samples. Variations in the application of fixatives (e.g. during trans-cardial perfusion of animals) or the potential loss of proteins from tissue sections during the various treatment steps can have a major impact on the consistency of immunolabelling [1]. This inherent variability of immunohistochemical approaches often hinders quantitative comparisons of protein expression levels in different brain samples.

Immunoblot analysis of tissue extracts obtained from dissected brain samples is widely used to study quantitative changes in protein levels. Electrophoretic separation of proteins and their subsequent transfer to blotting membranes allows the reliable and specific identification of immunoreactive bands with the correct molecular weights. While this approach is often used to validate the selectivity of antibody labelling, immunoblotting also has significant limitations. For example, (i) inaccuracies in the dissection of various brain regions, (ii) variability associated with lengthy and complex sample preparation procedures (e.g. tissue homogenisation, subcellular fractionation and protein extraction techniques), and (iii) proteolytic degradation of samples (e.g. due to post-mortem delays in human) can undermine spatial resolution, reproducibility and quantification.

To combine the fine anatomical resolution offered by conventional immunohistochemistry with optimal accessibility of immobilising matrix-bound proteins for immunochemical detection, various *in situ* blotting (histoblot) techniques were developed for the regional mapping of various protein targets [2–4]. These techniques are based on the direct transfer of native proteins from unfixed frozen tissue sections to an immobilising matrix (e.g. nitrocellulose transfer membrane). The blotted membranes can be subjected to similar immunochemical detection and quantification procedures that are widely used for conventional immunoblotting of electrophoretically separated proteins. These histoblots proved to be a convenient and highly reliable method for the mapping of neurotransmitter receptors (Fig. 1) [5–10], ion channels [11, 12] and other CNS proteins [13–15]. While the spatial resolution of histoblot technique is similar to radioligand autoradiography and *in situ* hybridisation histochemistry, it is not suitable for the investigation of protein distribution at the cellular and subcellular levels.

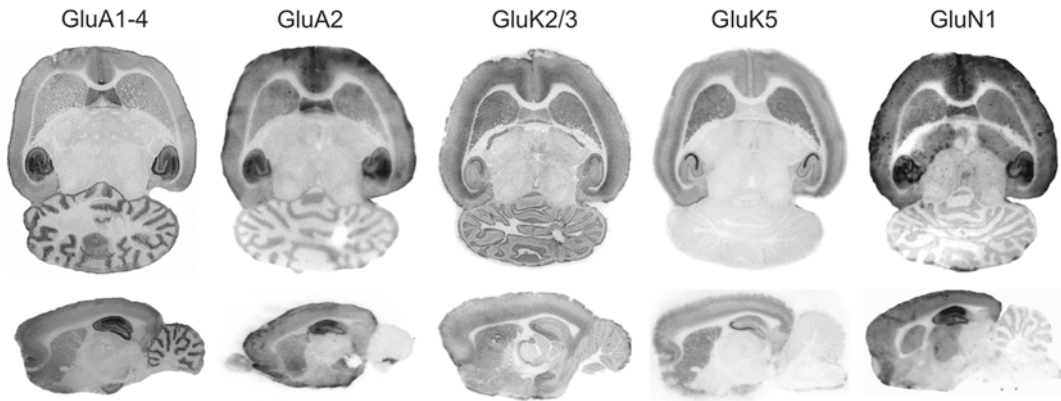


Fig. 1 Regional distribution of ionotropic glutamate receptor subunit proteins. Histoblots of horizontal (*top panels*) and sagittal (*bottom panels*) sections of adult rat brains were immunolabelled with affinity-purified GluA1-4 [16], GluA2, GluK2/3 (Abcam, Cambridge, UK), GluK5 [18] and GluN1 [16] subunit selective antibodies (Images were provided by Dr. Simon M. Ball)

This chapter describes the histoblot protocol that we have successfully applied for the mapping and quantitative comparisons of neurotransmitter receptor (Fig. 1) [9, 16–21] and ion channel [11] expression levels in different brain regions. The experimental procedures described below were initially used for studies of glutamate receptors in rodent brain samples at different developmental stages [22] or following the induction of seizures [23–26]. However, the same methodology and theoretical principles are also applicable to other protein targets in the CNS. Furthermore, the same methodology can also be used for the investigation of human brain samples (Fig. 2), which are often very challenging targets for conventional immunohistochemical or immunoblotting approaches.

2 Materials

2.1 Buffers

1. Transfer buffer: 48 mM Tris-base, 39 mM glycine, 2 % (w/v) sodium dodecyl sulphate (SDS), 20 % (v/v) methanol and pH \approx 10.5 (not adjusted). To prepare 100 mL of transfer buffer, dissolve 0.58 g Tris-base, 0.29 g glycine and 2 g SDS in deionised water and add 20 mL of methanol before volume is adjusted to 100 mL.
2. Tris buffer with saline and Tween 20 detergent (TBST buffer): 10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05 % (v/v) Tween 20. To prepare 1000 mL of TBST buffer, dissolve 1.21 g Tris-base, 8.77 g NaCl and 0.5 mL (or 0.552 g) Tween 20 and adjust pH to 8.0 using HCl before volume is adjusted to 1000 mL.

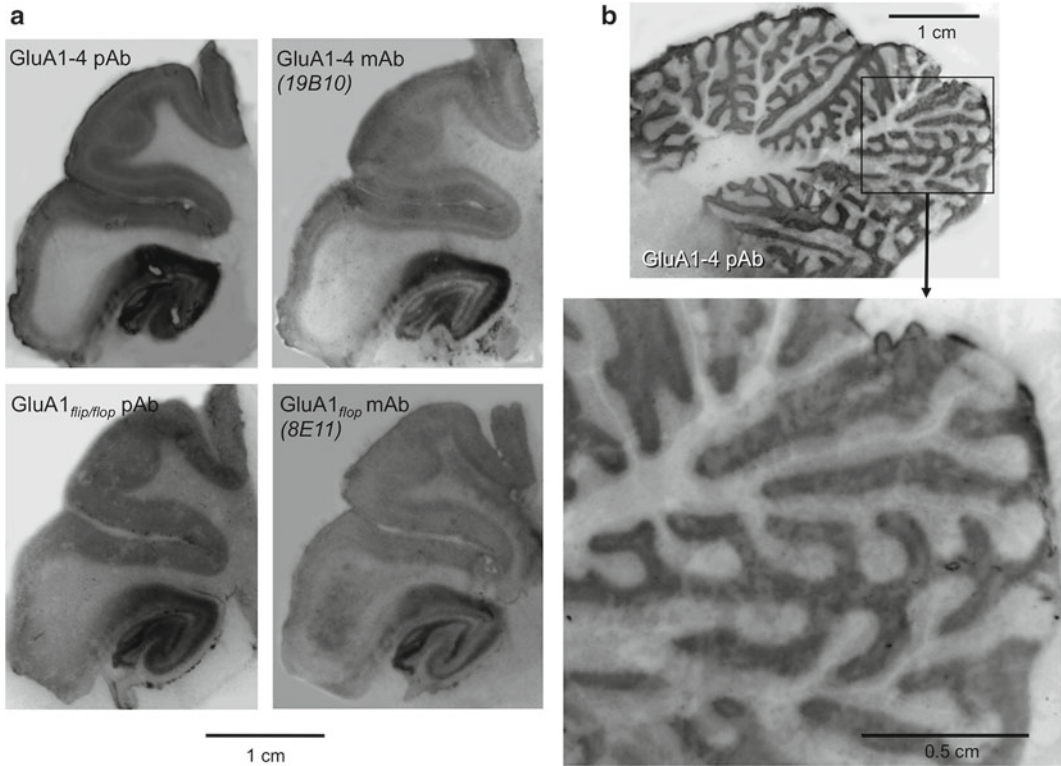


Fig. 2 Regional distribution of AMPA receptor subunits in human brain samples. Histoblots of human hippocampal (a) and cerebellar (b) sections were labelled with anti-GluA1-4 [16], anti-GluA1 *flip/flop* [27] polyclonal rabbit antibodies (*pAb*) or anti-GluA1-4 (19B10) and anti-GluA1 *flop* (8E11) monoclonal mouse antibodies (*mAb*) [9] to visualise the distribution of the corresponding subunit proteins

3. Blocking solution: 5 % (w/v) skimmed milk powder in TBST. Dissolve 5 g milk powder in 100 mL TBST.
4. Radioimmunoprecipitation assay buffer supplemented with ethylenediaminetetraacetic acid (EDTA) (RIPEA buffer): 20 mM Tris-HCl (pH 7.5), 60 mM NaCl, 0.4 % (v/v) Triton X-100, 0.1 % (w/v) SDS, 0.4 % (w/v) deoxycholic acid, sodium salt and 2 mM EDTA disodium dihydrate. To prepare 1000 mL of RIPEA buffer, dissolve 2.423 g Tris-base, 3.5 g NaCl, 4 mL (or 4.28 g) Triton X-100, 1 g SDS, 4 g deoxycholic acid, sodium salt and 0.7443 g EDTA disodium dihydrate in deionised water and adjust pH to 7.5 using HCl before volume is adjusted to 1000 mL.
5. Deoxyribonuclease I [EC 3.1.21.1] (DNase I) buffer: 100 mM Na-acetate (pH 5.0) and 5 mM MgSO₄. For 500 mL DNase I buffer, dissolve 6.8 g Na-acetate 3xH₂O (or 4.1 g anhydrous Na-acetate) and 0.616 g MgSO₄ 7xH₂O (or 0.301 g anhydrous MgSO₄) and adjust pH to 5.0 using acetic acid.

6. Strip buffer: 0.1 M Tris-HCl (pH 7.0), 2 % (w/v) SDS and 0.1 M β -mercaptoethanol. To prepare 200 mL of strip buffer, dissolve 2.423 g Tris-base and 4 g SDS in deionised water and adjust pH to 7.0 using HCl before volume is adjusted to 200 mL. Just before use, transfer solution into a fume hood and add 0.7 mL (or 0.78 g) β -mercaptoethanol (0.1 M final concentration).
7. Alkaline phosphatase (AP) buffer: 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 5 mM MgCl_2 . For 1000 mL AP buffer, dissolve 12.14 g Tris-base, 5.84 g NaCl and 1.01 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in deionised water and adjust pH to 9.5 using HCl or NaOH before volume is adjusted to 1000 mL.
8. AP developer: 0.33 % (w/v) nitro blue tetrazolium (NBT), 0.66 % (w/v) 5-bromo-4-chlor-3-indolyl phosphate (BCIP), 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 5 mM MgCl_2 .
 - 8.1. Prepare 5 % (w/v) NBT solution in 70 % (v/v) dimethylformamide (DMF) and 30 % (v/v) deionised water. For 200 μL NBT solution, use 0.01 g NBT, 140 μL DMF and 60 μL deionised water.
 - 8.2. Prepare 5 % (w/v) 5-bromo-4-chlor-3-indolyl phosphate (BCIP) solution in 100 % DMF. For 200 μL BCIP solution, dissolve 0.01 g BCIP in 200 μL DMF.
 - 8.3. AP developer should be prepared just before use: Add 16 μL of 5 % NBT and 33 μL BCIP solutions to 5 mL AP buffer and mix.
9. AP stopper (phosphate-buffered saline, PBS): 1.5 mM KH_2PO_4 , 8.5 mM Na_2HPO_4 (pH 7.5), 137 mM NaCl and 3 mM KCl. To prepare 1000 mL, dissolve 0.204 g anhydrous KH_2PO_4 , 1.207 g anhydrous Na_2HPO_4 (or 1.513 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), 8.01 g NaCl and 0.224 g KCl. Confirm that pH is 7.5 and adjust volume to 1000 mL.

3 Methods

3.1 Preparation of Brain Sections for Histoblots

Prepare 10 μm thick cryostat sections of frozen unfixed brains for histoblots. Trans-cardial perfusion of animals and cryoprotection of brain tissue are not necessary. The transfer of proteins from unfixed section onto nitrocellulose membranes will preserve anatomical organisation of brain samples without compromising antigenicity by fixation.

1. Anaesthesia: Follow national and international guidelines for the care and handling of animals. Use approved protocols for anaesthesia and killing of animals appropriate for the species used for experiments.

2. Freshly removed brains should be cleared of dura and surface blood vessels. Regions of interest need to be dissected from larger brains (e.g. human brain samples).
3. For freezing the brain tissue, use isopentane (at least 500 mL in a long beaker) prechilled to -35 – -40 °C on dry ice or in -80 °C freezer. Place the brain tissue onto aluminium foil and remove liquid using filter paper. Gently immerse the brain (without the aluminium foil) into the prechilled isopentane. The brain tissue can be damaged or deformed if there is not enough isopentane in the beaker, and the brain hits the bottom too soon. It takes about 5 min to completely freeze an adult whole rat brain in isopentane. For the removal of brains from the isopentane, use a chilled forceps to prevent its sticking to the brain tissue.
4. Frozen brains should be secured individually with prechilled soft paper towels in prechilled Falcon tubes (50 mL capacity), which can be used for storage and transportation. The brains should be stored at -80 °C or on dry ice during transportation.
5. Transfer samples to -20 °C freezer overnight before cryostat sectioning.
6. Use Tissue-Tek® O.C.T™ to attach brain samples to specimen chocks in the desired orientation and position. Keep samples at -20 °C.
7. Set cryostat chamber and block temperature to -20 °C, knife to -17 – -18 °C or use your own optimised temperature settings. Trim down brain samples to the region of interest and then collect 10 µm thick sections and thaw and mount them onto cleaned, uncoated glass microscope slides. Mount some of the sections onto poly-l-lysine or gelatin-coated glass slides for Nissl staining. Keep brain sections at -20 °C until they are processed for histoblotting or at -80 °C for longer-term storage.

3.2 Transfer of Proteins from Brain Sections onto Nitrocellulose Membranes

Use a pair of powder-free gloves to avoid contamination of the membranes by fingerprints.

1. Cut Whatman filter paper (Nr. 1001917 or Nr. 3030917) to the appropriate size (about 3×3 cm for rat brains).
2. One by one dip both ends of the filter papers into transfer buffer (48 mM Tris-base, 39 mM glycine, 2 % (w/v) SDS, 20 % (v/v) methanol, $\text{pH} \approx 10.5$) and lay them onto a clear glass surface without trapped air bubbles. Build up three layers of moist filter papers and put dry filter papers immediately next to them (both sides) to remove any excess liquid. Papers should not release buffer even under pressure.
3. Use the appropriate size (e.g. 2.4×2.4 cm for rat brain) of nitrocellulose membrane paper (e.g. Schleicher & Schuell, BA

85, 0.45 μm , Nr 401196). Proteins should be transferred onto the outer (convex) surface in the roll. This surface needs to be marked with an identification number of the section using a pencil at one of its corners. Do not use pen.

4. Fully dip the nitrocellulose membrane into transfer buffer and layer the membrane on the top of the stack of wet filter papers without trapped air bubbles.
5. Without horizontal movement, carefully place the glass slide with the brain section facing down onto the nitrocellulose membrane in a way that no air bubbles remain.
6. Apply a weight (e.g. inverted full 1000 mL laboratory glass bottle with plastic top on) gently from one side to remove trapped air and leave it for 30 s without any movement.
7. Immerse glass side with the attached nitrocellulose membrane facing down into blocking buffer (5 % (w/v) skimmed milk powder in TBST) and leave it for one hour without agitation. Nitrocellulose membrane will normally detach from glass slide. If not, peel it off gently using a forceps. Discard used filter papers; you need to prepare new ones for each transfer to avoid contamination of the nitrocellulose membranes.
8. Transfer nitrocellulose membranes into appropriate size petri dishes (or 6 well plates) filled with blocking buffer.
9. Prepare DNase I solution (you will need 2 mL/well): add 10 Units of DNase I stock (RNase-free) to 2 mL DNase I buffer.
10. Rinse membranes in TBST once for 1 min. From this point samples should be placed on a shaker for subsequent steps to make sure that the whole membrane is evenly exposed to buffers and reagents.
11. Incubate nitrocellulose membranes in DNase I solution at 37 °C for 20 min. The enzymatic degradation of DNA will improve access to membrane-bound protein epitopes.
12. Wash nitrocellulose membranes in RIPEA for 20 min.
13. Wash nitrocellulose membranes in TBST for 2 \times 10 min.
14. Add β -mercaptoethanol to strip buffer under fume hood (you will need 1 mL/well).
15. Remove TBST and add strip buffer (1 mL/well) to nitrocellulose membranes under fume hood. Seal petri dishes (or plates with lids) tightly using parafilm and incubate at 45 °C for 1 h. This step will remove adhering tissue residues from the nitrocellulose membrane.
16. Remove strip buffer and wash nitrocellulose membranes in RIPEA buffer for 10 min.
17. Wash in TBST for 2 \times 5 min.
18. Incubate in blocking solution for 1 h.

3.3 Immunolabelling

19. Dilute primary antibody in blocking solution (200 μL /blot). While optimum concentration needs to be determined for each primary antibody, 1 $\mu\text{g}/\text{mL}$ is usually a good starting point for affinity-purified antibodies.
20. Seal nitrocellulose membranes with primary antibodies into small plastic pockets (prepare these just slightly bigger than the nitrocellulose membrane) using an impulse sealer and incubate samples at 4 $^{\circ}\text{C}$ overnight on shaker or rotating wheel in cold room.
21. Remove membranes from plastic pockets and place them in RIPEA-containing petri dishes for washing for 20 min. Use TBST instead of RIPEA if the expected immunolabelling is weak.
22. Wash in blocking solution for 2×10 min.
23. Dilute alkaline phosphatase-labelled secondary antibody in blocking solution (e.g. 1:5000 dilution of donkey anti-rabbit IgG-AP, Promega; 1 mL/well). Add 1 mL of diluted secondary antibody to membranes and incubate on shaker for 90 min at room temperature ($\sim 20^{\circ}\text{C}$).
24. Wash membranes in RIPEA for 20 min. Use TBST instead of RIPEA for this step if the expected immunolabelling is weak.
25. Wash membranes in TBST for 2×10 min and rinse it one more time in TBST briefly.
26. Prepare AP developer as described above and add 1 mL/well.
27. Develop alkaline phosphatase reaction at room temperature ($\sim 20^{\circ}\text{C}$) as long as required. Usually it takes about 5–30 min to get clear labelling. Leave it at 4 $^{\circ}\text{C}$ overnight if there is no detectable labelling after 30 min.
28. Stop AP reaction using PBS and rinse membranes three times in fresh PBS.
29. Store membranes in PBS at 4 $^{\circ}\text{C}$ until scanning or photography. Do not dry membranes before images are captured because labelling will fade.

4 Notes

1. Using temperatures below -40°C for the freezing of brain samples may result in the fragmentation of the frozen brain tissue or difficulties with cutting of sections using cryostat. Following appropriate freezing, even relatively large brain areas (e.g. cryostat sections of whole human cerebellum, hippocampus, striatum, etc.) can be processed for histoblotting (Fig. 2).

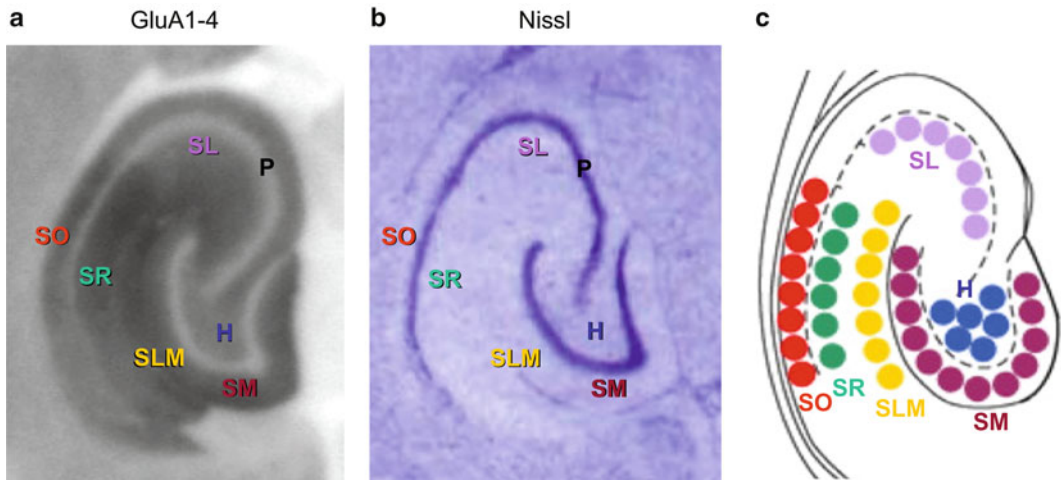


Fig. 3 Illustration of the sampling method used to compare immunoreactivities in different hippocampal regions. (a) Hippocampal region of a rat brain histoblot immunolabelled with a rabbit polyclonal anti-GluA1-4 antibody [16]. (b) Nissl-stained sections facilitate the identification of different brain regions after immunostaining for sampling of immunoreactivity. (c) The colours on the schematic diagram represent layers of the hippocampal formation: *red* stratum oriens (SO), *green* stratum radiatum (SR), *yellow* stratum lacunosum-moleculare (SLM), *plum* stratum moleculare of dentate gyrus (SM), *blue* hilum of dentate gyrus (H), *lilac* stratum lucidum of CA3 (SL). Density readings can be taken by placing open circular cursors with a diameter of 0.1 mm at the indicated adjacent positions along SO (8 circles), SR (6 circles), SLM (7 circles), SM (12 circles), H (6 circles), SL (7 circles). See text and Refs. [11, 19, 20, 22–26] for details and examples

2. Accurate setting of the thickness of the cryostat sections is essential for quantitative comparisons. Based on our experience, 10 μm thick sections (that likely to represent a single cell layer) are ideal for histoblots. This is consistent with previous studies [3]. Because the unfixed frozen brain sections are also suitable for the study of radioligand binding sites using autoradiography and for in situ hybridisation histochemistry analysis of mRNA distribution, these can be performed in parallel to complement the histoblot mapping of the corresponding proteins.
3. Nissl staining of adjacent sections can help with the accurate identification of immunopositive brain structures (Fig. 3). For Nissl staining use poly-l-lysine or gelatin-coated glass slides. After overnight drying at room temperature ($\sim 20^\circ\text{C}$), wash slides in 70 % (v/v) ethanol for 2 min. Use 1 % (w/v) aqueous cresyl violet for 5 min. Remove excess stain by washing in tap water. Wash slides in 10 % (v/v) methylated spirits containing a few drops of acetic acid. Wash slides in methanol three times and once in xylene before mounting in DPX mountant (BDH Laboratory Supplies, Poole, UK) for microscopy or scanning.

4. Nitrocellulose membranes with transferred brain proteins (after **step 18** under 3.2) can be stored at $-20\text{ }^{\circ}\text{C}$ for several weeks without deterioration of antigenicity and subjected to immunostaining later. For storage, place blotted nitrocellulose membranes between two layers of filter papers (e.g. Whatman filter paper Nr. 1001917 or Nr. 3030917) pre-soaked in blocking buffer. Seal nitrocellulose membranes with filter papers into small plastic pockets using an impulse sealer and store them at $-20\text{ }^{\circ}\text{C}$.
5. Optimum dilution and incubation conditions need to be established for each primary and secondary antibody. Most affinity-purified primary antibodies produce good immunolabelling at $1\text{ }\mu\text{g}/\text{mL}$ concentration following overnight incubation at $4\text{ }^{\circ}\text{C}$. Alkaline phosphatase-conjugated secondary antibodies at 1:5000 dilution, applied for 90 min at room temperature (or overnight at $4\text{ }^{\circ}\text{C}$), are generally suitable for the visualisation of primary antibodies. While the use of horseradish peroxidase-conjugated secondary antibodies in combination with enhanced chemiluminescence is frequently used to amplify signal for conventional immunoblots, this detection system reduces the resolution of histoblots therefore less suitable than the above-described alkaline phosphatase-based visualisation of immunoreactivity.
6. Primary antibodies that produce specific labelling on immunoblots often fail to recognise the same protein targets in fixed tissue samples using conventional immunohistochemistry. Because the presentation of proteins in histoblots is very similar to conventional immunoblots, antibodies are more likely to work in both techniques. However, the use of appropriate positive and negative controls is required to confirm the specificity of immunolabelling obtained with histoblots [1]. Omitting the primary antibody and using sera obtained from immunised animals before the first injection of the antigen ('pre-immune serum') instead of the primary antibody are frequently used negative controls. In addition, primary antibodies should be preabsorbed with excess of the corresponding antigenic peptide or protein. Under these conditions, the labelling of endogenous proteins presented on histoblots is blocked by the saturation of antibodies with the antigens. While this control will show that the antibody binds to the antigen, it does not exclude the possibility of cross-reactions with other proteins. It is often very helpful to use two different antibodies raised against different epitopes of the same target protein. If patterns of staining are the same with both antibodies, this is strong circumstantial evidence in favour of specificity [1, 17]. The ideal control sample is tissue obtained from a transgenic animal, which lacks the target antigen. Comparison of the

distribution of immunoreactivity with images obtained by using other localisation techniques (e.g. autoradiography using radioligands or in situ hybridisation histochemistry) could provide additional supporting evidence. For example, areas where the presence or absence of the target antigen is expected based on these complementary imaging approaches can be used as positive and negative controls, respectively, [1].

7. While in most histoblot experiments 5 % (w/v) skimmed milk powder containing blocking solution is able to minimise non-specific labelling, normal serum or fish skin gelatin (3–5 % w/v) can be used as an alternative if the background labelling is high. Unlike normal serum, bovine serum albumin (BSA) or milk, fish gelatin does not contain IgG or serum proteins that could cross-react with mammalian antibodies. Also, fish gelatin effectively blocks non-specific binding sites on nitrocellulose membranes and suitable for the dilution of antibodies during immunostaining procedures [9].
8. Digital greyscale images of histoblots can be produced by standard desktop scanners, which provide even and consistent illumination of nitrocellulose membranes even if several are scanned at the same time for quantitative comparisons. Membranes should remain wet during scanning to prevent the fading of immunopositive areas. Therefore, wet nitrocellulose membranes should be covered with a transparent plastic sheet after they are laid on the glass surface of the scanner. In addition to keeping the membranes wet, the plastic sheet also helps with the removal of trapped air bubbles from under the nitrocellulose membranes.
9. Image analysis and processing can be performed using Adobe® Photoshop® (Adobe Systems, San Jose, CA, USA) or Image J (NIH Image, Bethesda, MD, USA; <http://imagej.nih.gov/ij>) software. Greyscale images need to be captured and treated identically to allow comparison of pixel densities (arbitrary units) of immunoreactivity in different brain regions. The pixel density can be measured using open circular cursors with a set diameter (e.g. 0.1 mm), which is appropriate for the region of interest (Fig. 3). The cursors should be placed in different brain regions identified based on adjacent cresyl violet-stained sections [11, 19, 20, 23]. For example, different regions of the hippocampus can be analysed by placing circular cursors with a diameter of 0.1 mm at adjacent positions along the *stratum oriens*, *stratum radiatum*, *stratum lacunosum-moleculare*, *stratum moleculare* of dentate gyrus, *hilum* of dentate gyrus and *stratum lucidum* of CA3 (Fig. 3). The same approach can be applied to other brain regions. About ten different background determinations need to be performed near the brain protein containing areas of the immunostained nitrocellulose membranes. The average of these

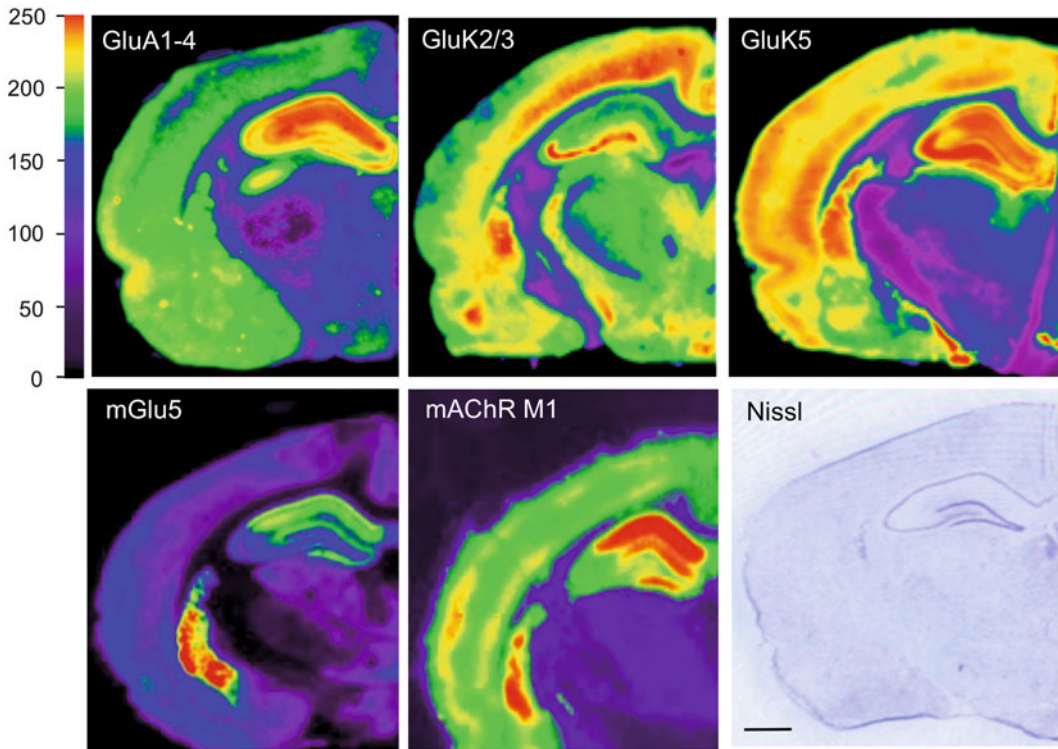


Fig. 4 Colour gradients illustrate the regional expression profiles of different neurotransmitter receptor proteins. Histoblots of coronal rat brain sections were immunostained for either ionotropic glutamate receptor subunits GluA1-4 [16], GluK2/3, GluK5 (Abcam, Cambridge, UK), metabotropic glutamate receptor isoform 5 (*mGlu5*) or muscarinic acetylcholine receptor M1 (*mAChR M1*) [20]. Greyscale histoblot images were converted to colour gradients using gradient mapping [19–21]. Scale bar, 1 mm (Images were provided by Dr. Simon M. Ball)

background values needs to be subtracted from the average pixel densities measured within various brain regions. Following background corrections, the average pixel density for the whole region from one animal should be counted as one ‘n’. Differences between corresponding brain regions of different animals should be assessed using a two-way analysis of variance (ANOVA) and further compared with the Bonferroni post hoc test, at a minimum confidence level of $p < 0.05$.

10. For illustration purposes greyscale histoblot images can be converted to colour gradients using the gradient mapping function of Adobe® Photoshop® (Adobe Systems, San Jose, CA, USA) [19–21] (Fig. 4).

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