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Differential expression of AMPA glutamate receptor mRNAs in the rat adrenal gland

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Localisation of mRNA encoding the four AMPA glutamate receptor subunits in the rat adrenal gland was investigated using in situ hybridisation. GluR1 is found in the zona glomerulosa of the cortex, GluR3 in the remaining parts of the cortex, GluR2 in adrenal medullary cells and GluR4 at a very low level in the zona glomerulosa. All four receptor mRNAs are found in medullary ganglion cells. The flip form of GluR2 and GluR3 dominate and the GluR2 mRNA is present in the arginine encoding form. Different cell populations of the adrenal gland may express homomeric forms of different receptor subtypes.

Glutamate receptor; AMPA; mRNA expression; Hybridisation, in situ; Adrenal gland; Rat

1. INTRODUCTION

Glutamate is the major excitatory neurotransmitter of the central nervous system and glutamate receptors may be divided into ion-channel forming (ionotropic) and G-protein coupled (metabotropic) receptors. The ionotropic glutamate receptors are a family of ligand-gated ion channels that may be further subdivided into NMDA and non-NMDA receptors [1,2]. In the latter group the best characterised members are the family of AMPA receptors, and currently four structurally related molecular subunits are known in this family [3–6]. The combination of subunits in the receptor complex determines the functional properties of the channel; when the GluR2 subunit is included, the formed channels will have a low permeability to calcium ions [7–10]. The four AMPA subunits have been labelled GluR1–4, GluRA-D or α1-α4. In this presentation the numbering GluR1-GluR4 will be used. Until now, the AMPA receptors have only been demonstrated in the central nervous system [3–6] and in the retina [11,12].

2. MATERIALS AND METHODS

Male Sprague–Dawley rats (weighing appox. 200 g) were used. Adrenal glands were removed after perfusion fixation of the animal with freshly prepared 4% paraformaldehyde and processed as previously described [13], except that isopentane cooled on dry-ice, was used for freezing instead of Freon 22. Preparation of ^{35}S-labelled sense- and anti-sense RNA probes and in situ hybridisation were done as described [13], except that the proteinase K step was omitted, the final concentration of radioactivity in the hybridisation solution was 50.000 cpm/ml, hybridisation was done at 57°C and washing of sections in 50% formamide solution at 62°C and 67°C. Following exposure to beta-max film (Amersham, UK), sections were coated with emulsion and exposed for 29 days.

The following cDNA fragments coding for the N- terminal parts of the four rat AMPA glutamate receptors were isolated by PCR [14] based on the published sequence [5], inserted into pBluescript vector, analysed by DNA sequencing and used for probe preparation: GluR1(bp. no. 1–850), GluR2(bp. no. 1–934), GluR3(bp. no. 1–756) and GluR4(bp. no. 1–1058).

RNA was prepared from freshly removed or frozen rat tissue using the thiocyanate-phenol extraction method [15] as modified for use with the RNaid kit (Bio101): 2.5 ml of cell lysis solution was used lyse 4 adrenal glands and these were homogenized on ice in a Dounce glass homogenizer. Consecutive extractions were performed with 2.5 ml of acid phenol and 2 × 1 ml of chloroform/isoamylalcohol (25:1). The remaining part of the procedure was as recommended by the manufacturer. Finally the RNA was dissolved in water and the absorption at 260 and 280 nm. measured. Only RNA with an OD ratio 260 nm/280 nm of > 1.95 was used.

cDNA was synthesised from 4 mg of total RNA using Moloney murine leukemia virus reverse transcriptase primed with 0.2 mg of random hexamers in a reaction mixture of 45 mM Tris (pH 8.3), 68 mM KCl, 15 mM dithiothreitol, 9 mM $MgCl_2$, 0.08 mg/ml bovine serum albumin and 1.8 mM of each deoxynucleotide for 60 min at 37°C. The entire cDNA synthesis reaction was used for PCR after diluting 3-fold with water and addition of 60 pmol of each primer. Taq polymerase (2 units) was added after denaturation and equlibration at the annealing temperature. For analysis of the flip/flop region amplification was for 35 cycles at 95°C/55°C/ 37°C (each for 1 min) using pairs of the following 21- mer oligonucleotides: GluR1: 5′ position 2074 (5′- GAACCATCCGTGTTTGTTCTT) and 3′ position 2541 (5′-ATGGCTTCATTGATGGATTGC); GluR2 80: 5′ position 2070 (5′-AATGTGGACTTATATGAGGAG) and 3′ position 2558 (5′-AGGAAGATGGGTTAATATTCT); GluR3: 5′ position 2109 (5′-AGAGCCATCTGTGTTTACCAA) and 3′ position 2556 (5′-TTTTGGGTGTTCTTTGTGAGT); GluR4: 5′ position 2074 (5′-ATGTGGACCTACATGCGATCG) and 3′ position 2557 (5′- GGCTTTGT-

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate; GluR, glutamate receptor; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction.

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TTCTTGTGGCTTC). For analysis of GluR2 posttranscriptional editing PCR amplification was done for 35 cycles at 95°C/56°C/37°C (each for 1 min) using the oligonucleotides: 5'-GGAATGGTATG-GTTGGAGAGC and 5'-AGTTAGCCGTGTAGGAGGAGA. DNA sequencing of the PCR fragment purified by Geneclean (Bio 101) was done using Sequenase (USB) according to manufacturers recommendations.

3. RESULTS AND DISCUSSION

In situ hybridisation to sections of rat adrenal medulla showed a differential pattern of expression for the four AMPA glutamate receptors (Fig. 1), with a strong signal using the anti-sense RNA probes, and lack of signal using RNA transcribed from the complementary strand, except for the unspecific binding observed to a blood vessel in the peri-adrenal fat (Fig. 1). The strongest expression was seen for the GluR3 (Fig. 1e). Examination at the histological level (Fig. 2) confirmed a strong signal in most cortical cells, except those of the zona glomerulosa (Fig. 2a and c). The cells of the cortex zona glomerulosa showed signal for the GluR1 (Figs. 1a and 2b), while all medullary cells showed signal for GluR2 mRNA (Figs. 1c and 2d). In the medulla, the sympathetic ganglion cells, discernible by their size showed positive signal for all four receptors (Fig. 3). A weak signal could be seen for the GluR4 mRNA in the outer layer of the cortex (Fig. 1g). To further characterise the mRNA present in the adrenal gland, mRNA was purified, cDNA synthesised after random priming and PCR amplification performed. The oligonucleotides used for the PCR were selected to amplify the region that is alternatively spliced in the receptors to yield the two different flip/flop forms of the receptor mRNA [16] and to avoid cross-amplification between receptor subtypes. The oligonucleotides were further selected so that a restriction endonuclease site specific for the flip or flop region would not be present in the remaining fragment. Cutting of the fragment with this restriction endonuclease could then give information on whether the flip or the flop version of the mRNA was found. Under the annealing conditions used (57°C), amplification from rat adrenal mRNA only showed the approx. 500 bp fragment when using the oligonucleotide pairs for GluR2 and GluR3, presumably because of to low amount of the GluR1 and GluR4 mRNA being present in the total adrenal gland mRNA. Together with similar fragments amplified from cerebral cortex and cerebellum RNA these fragments were cut with restriction endonucleases, precipitated with ethanol and analysed by agarose gel electrophoresis (Fig. 4). The cerebral cortex mRNA and cerebellar mRNA contain both flip and flop forms as previously reported [17], while the adrenal gland mRNA contains mainly the flip form of the mRNA. In addition to the flip/flop variation the GluR2 mRNA is posttranscriptionally modified to insert a codon for arginine in the second transmembrane domain, determining the calcium permeability of the

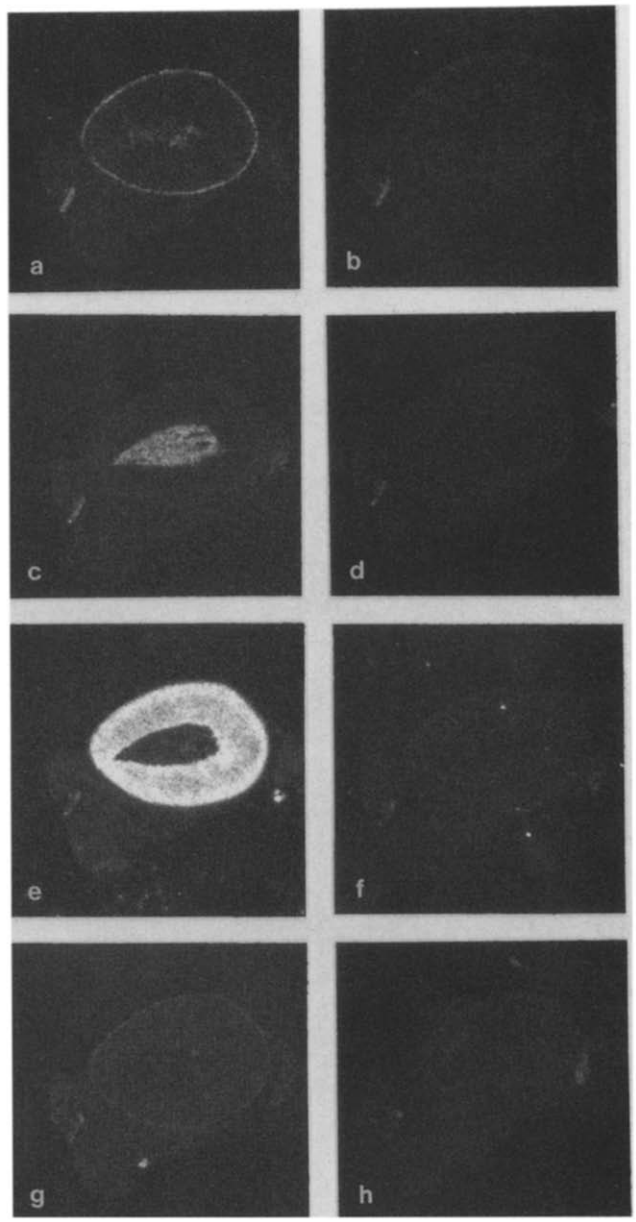

Fig. 1. Film images of rat adrenal gland sections hybridised using anti-sense (a, c, e, g) and sense RNA probes (b, d, f, h) to the four different AMPA glutamate receptors: GluR1 (a, b), GluR2 (c, d), GluR3 (e, f) and GluR4 (g, h). Micrographs prepared from beta-max film exposed for 5 days. Magnification 5.9 ×.

formed channel [8]. To determine which form is found in the adrenal medulla a 459 bp fragment was amplified from cDNA synthesised from adrenal gland mRNA (result not shown). The DNA sequence of this region of the PCR fragment was determined. and found to be identical to the edited version reported [8]. At least 80% of the adrenal gland GluR2 mRNA thus encodes a presumed low-calcium-permeable receptor.

The binding of [^{3}H]glutamate to the adrenal gland has previously been reported in a number of different

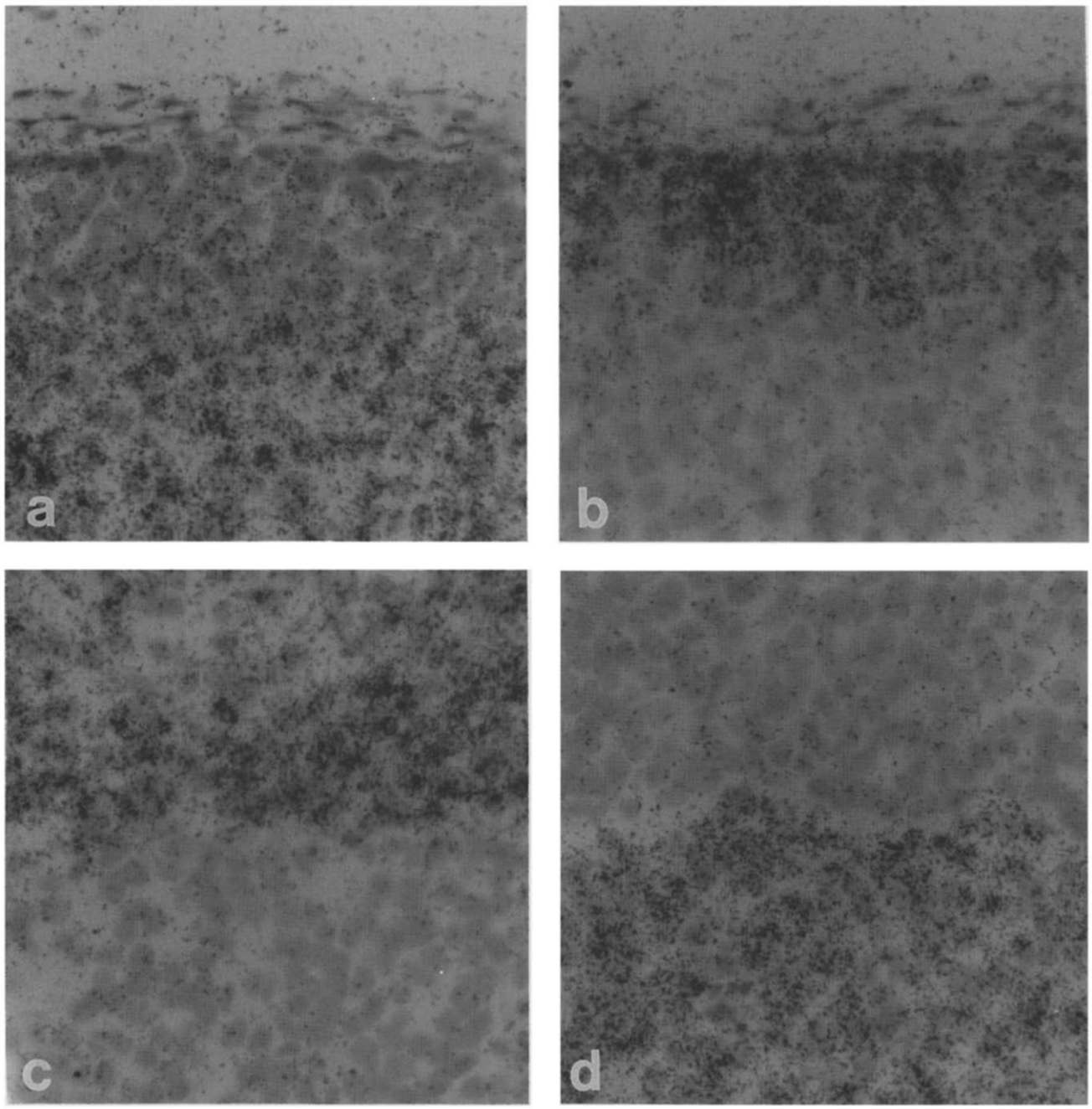

Fig. 2. Micrographs demonstrating the cellular localisation of the hybridisation signal for GluR3 (a, c), GluR1 (b) and GluR2. Note the presence of signal for GluR1 in the pars glomerulosa of the outer adrenal cortex (b), the finding of GluR3 mRNA in most but not all cells of the adrenal cortex (b, c) and the uniform distribution of gluR2 mRNA in medullary cells (d). Magnification 784 ×.

species, including the rat. The reports are contradictory with respect to the pharmacological profile of displacement of the [^{3}H]glutamate binding and to whether stimulation with glutamate may affect release of catecholamines [18–21].

Our findings strongly support the presence of ionotropic glutamate receptors in the rat adrenal gland, and further indicate that in contrast to most regions of the brain, homomeric AMPA glutamate receptors, composed only of one subtype, are likely to be formed in this tissue. Future experiments using subtype selective antibodies and possibly electrophysiological measurements on adrenal gland cells will reveal if this is the case.

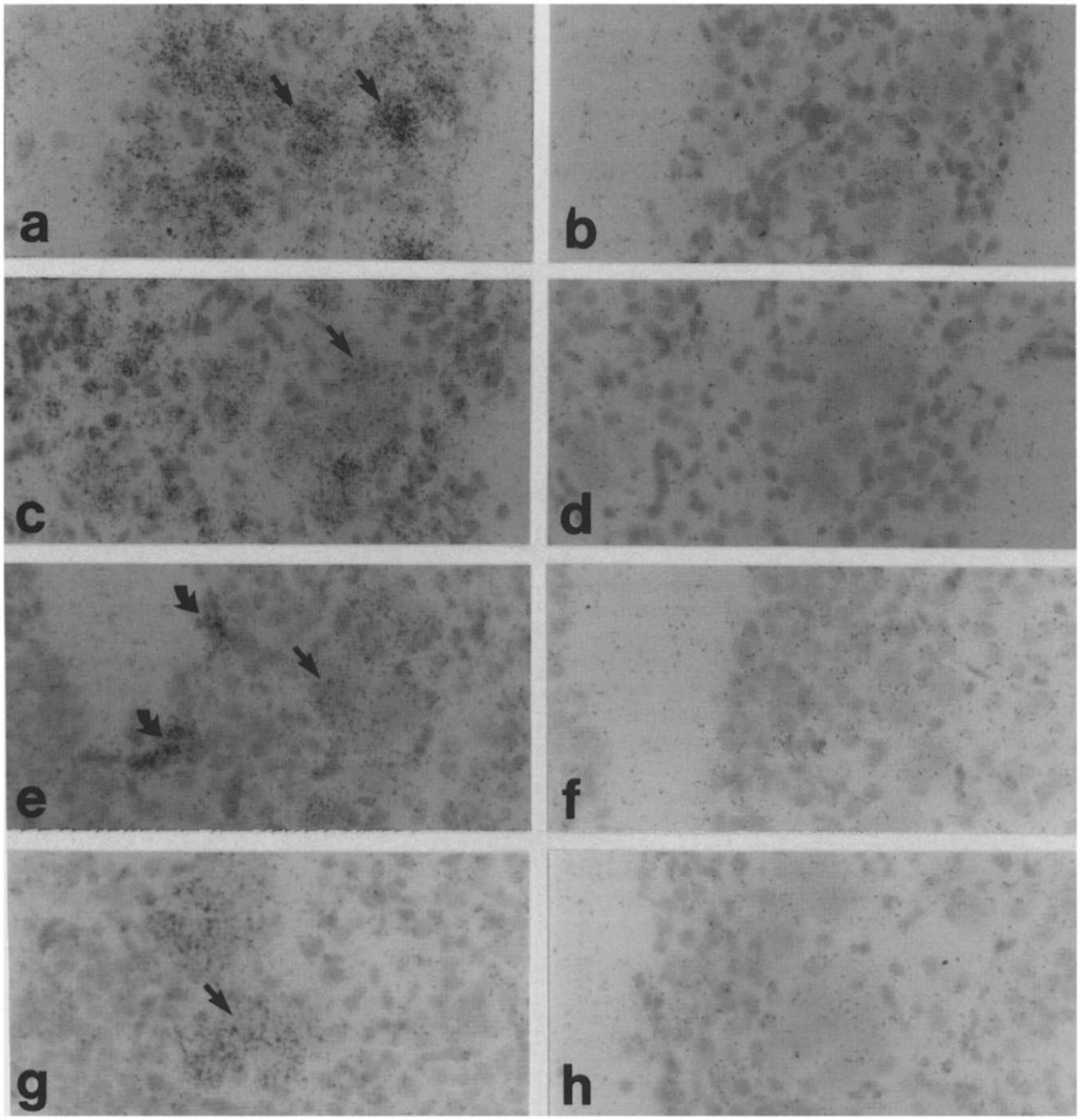

Fig. 3. Micrographs using anti-sense (a, c, e, and g) and sense RNA probes (b, d, f, h) to the four different AMPA glutamate receptors in rat adrenal medulla: GluR1 (a, b), GluR2 (c, d), GluR3 (e, f) and GluR4 (g, h). Note the signal over medullary ganglion cells for all four receptor subtypes (straight arrows in a, c, e and g) and the presence of a few small medullary cells showing signal for GluR3 in the medulla (curved arrows in e). Magnification 284 ×.

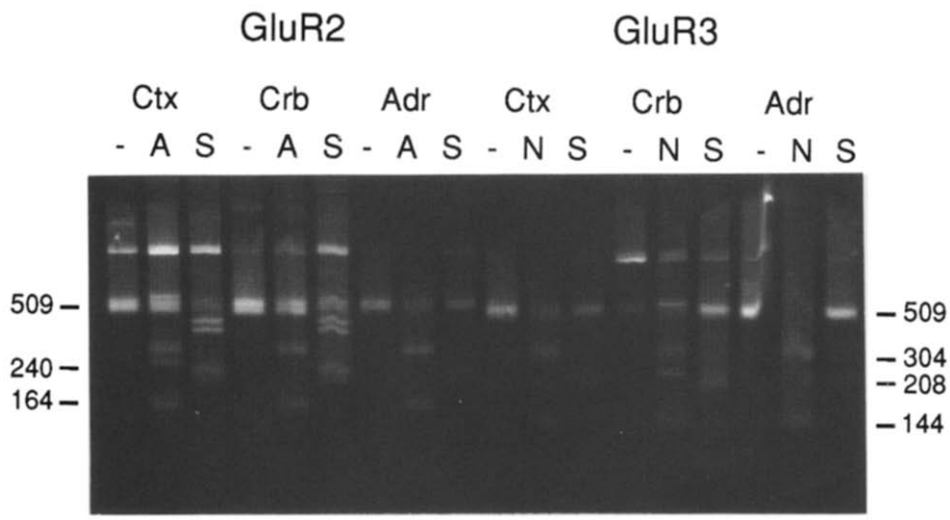

Fig. 4. Restriction enzyme analysis of ^{32}P-labelled PCR amplification products prepared from cerebral cortex, cerebellar or adrenal RNA using oligonucleotides flanking the flip/flop area of GluR2 and GluR3. Enzyme symbol explanation: -, no enzyme; A, *AvaI*; S, *StuI*; N, *NciI*. The presence of an *AvaI* (in GluR2) or *NciI* (in GluR3) site demonstrates the flip form, while *StuI* (in both GluR2 and GluR3) demonstrates the flop form. Sizes of expected bands are indicated in basepairs.

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