# Neuronal and Glial Localization of NR1 and NR2A/B Subunits of the NMDA Receptor in the Human Cerebral Cortex

Fiorenzo Conti, Paolo Barbaresi, Marcello Melone and Alessandro Ducati[1]

Institute of Human Physiology and [1]Neurosurgical Clinic, University of Ancona, Ancona, Italy

***N*-Methyl-D-aspartate (NMDA) receptors play a critical role in many cortical functions and are implicated in several neuropsychiatric diseases. In this study, the cellular expression of the NMDAR1 (NR1) and NMDAR2A and B (NR2A and B) subunits was investigated in the human cerebral cortex by immunocytochemistry with antibodies that recognize the NR1 or the NR2A and B subunits of the NMDA receptor. In frontal (areas 10 and 46) and temporal (area 21) association cortices and the cingulofrontal transition cortex (area 32), NR1 and NR2A/B immunoreactivity (ir) were similar and were localized to numerous neurons in all cortical layers. NR1- and NR2A/B-positive neurons were mostly pyramidal cells, but some nonpyramidal neurons were also labeled. Electron-microscopic observations showed that NR1 and NR2A/B ir were similar. In all cases, labeling of dendrites and dendritic spines was intense. In addition, both NR1 and NR2A/B were consistently found in the axoplasm of some axon terminals and in distal astrocytic processes. This investigation revealed that numerous NMDA receptors are localized to dendritic spines, and that they are also localized to axon terminals and astrocytic processes. These findings suggest that the effects of cortical NMDA activation in the human cortex do not depend exclusively on the opening of NMDA channels located at postsynaptic sites, and that the localization of NMDA receptors is similar in a variety of mammalian species.**

## Introduction

The *N*-methyl-D-aspartate (NMDA) receptor, one of the ionotropic glutamate receptor subtypes, has been the focus of much experimental work for its unique mode of operation at central nervous system synapses, for its reported role in sensory transmission, synaptic plasticity, learning and memory, and circuit development, and for its alleged involvement in numerous neuropsychiatric disorders (Ascher *et al.*, 1991; Nelson and Sur, 1992; Bliss and Collingridge, 1993; Daw *et al.*, 1993; Collingridge and Watkins, 1994; Scheetz and Constantine-Paton, 1994; Mori and Mishina, 1995; Conti and Hicks, 1996; Tsien *et al.*, 1996).

Molecular cloning has shown that NMDA receptors are formed by subunits belonging to the NMDAR1 (NR1) and NMDAR2 (NR2) classes in both rodents (Monyer *et al.*, 1992; Nakanishi, 1992; Cik *et al.*, 1993; Sheng *et al.*, 1994; Sucher *et al.*, 1996) and humans (Karp *et al.*, 1993; Planells-Cases *et al.*, 1993; Foldes *et al.*, 1994; Le Bourdèlles *et al.*, 1994; Adams *et al.*, 1995; Zimmer *et al.*, 1995; Hess *et al.*, 1996), with high interspecific homology (>90–95% respectively). NR1 has the basic features of the NMDA receptor (Moriyoshi *et al.*, 1991), it can exist in several isoforms generated by alternative splicing (Zukin and Bennett, 1995), and is the essential subunit of the NMDA receptor, as shown by the abolishment of classical NMDA neuronal responses following targeted disruption of the NR1 gene (Forrest *et al.*, 1994; Li *et al.*, 1994). The NR2 class includes four different subunits, NR2A-D, encoded by separate genes (Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993). *In situ* hybridization has shown that these subunits are expressed differently in the brain (Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993; Watanabe *et al.*, 1993) and during development (Monyer *et al.*, 1992; Farrant *et al.*, 1994; Sheng *et al.*, 1994). Electrophysiological studies have shown that NR2 subunits produce detectable currents only when they are coexpressed with NR1 (Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993), and that when NR1 is combined with different NR2 subunits, both electrophysiological and pharmacological responses are modified (Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992, 1994; Nakanishi, 1992; Stern *et al.*, 1992; Ishii *et al.*, 1993; Williams *et al.*, 1993, 1994; Hollmann and Heinemann, 1994; McBain and Mayer, 1994; Flint *et al.*, 1997; Anson *et al.*, 1998; see also Buller *et al.*, 1994).

Recent insights into the molecular biology of NMDA receptors have contributed to the generation of potent and specific tools for studying the localization of these receptors at the cellular level. In several laboratories, including ours, these tools have been employed to study the cellular localization and distribution of NMDA receptors in rat and monkey neocortex, one of the brain regions where the role of NMDA receptors appears particularly well documented (Conti and Hicks, 1996). These studies have shown that: (i) NMDA receptors are present in many but not all cortical neurons (Aoki *et al.*, 1994; Conti *et al.*, 1994; Huntley *et al.*, 1994; Conti and Minelli, 1996) and that neurons expressing NMDA receptors appear to be less numerous in layer IV than in layers II–III and V–VI (Conti and Minelli, 1996); (ii) in cortical neurons NMDA receptors are mostly formed by NR1 and NR2A and/or B subunits (Moriyoshi *et al.*, 1991; Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992, 1994; Nakanishi, 1992; Ishii *et al.*, 1993; Wenzel *et al.*, 1995; Conti *et al.*, 1997; Scherzer *et al.*, 1998); and (iii) the vast majority of NMDA receptors are located postsynaptically on dendrites and dendritic spines (Aoki *et al.*, 1994; Huntley *et al.*, 1994, 1997; Petralia *et al.*, 1994a,b; Conti and Minelli, 1996; Conti *et al.*, 1996, 1997; De Biasi *et al.*, 1996). Based on this evidence, the bulk of the effects of NMDA receptor activation appear to be generated at distal dendrites and dendritic spines.

The results of some recent investigations, however, suggest that NMDA-mediated processes may also be due to the activation of NMDA receptors located at non-conventional sites. Indeed, it has been shown that: (i) some NMDA receptors are presynaptic hetero- and autoreceptors, as NR1- and NR2A/B-immunoreactivity (ir) can be observed at axon terminals forming both asymmetric and symmetric synapses (De Biasi *et al.*, 1996; see also Aoki *et al.*, 1994; Huntley *et al.*, 1994; Johnson *et al.*, 1996); and (ii) NMDA receptors are also expressed by astrocytes (Conti *et al.*, 1996).

Whether these data also apply to the human cerebral cortex is at present a matter of mere conjecture, as the only available

**Table 1**
Summary of clinical data

| Patient reference | Age (years)/sex | Major symptoms | Pathology | Drug/daily dose/duration (days) |
|---|---|---|---|---|
| HBC970105 | 56/F | high intracranial pressure | hemorrhage from capsulo-striatal cavernoma | Phenobarbital/100 mg/35 |
| HBC970507 | 53/F | visual field defect | temporal fossa meningioma | Carbamazepine/400 mg/30 |
| HBC970508 | 32/M | oculomotor palsy | posterior fossa hemangio-pericytoma (transzygomatic subtemporal approach) | no therapy (refused) |
| HBC980510 | 60/M | high intracranial pressure | frontal meningioma | Valproate/1000 mg/30 |
| HBC980611 | 64F | high intracranial pressure | fronto-orbital meningioma | Barbesaclone/100 mg/30 |

information on the expression of NMDA receptor subunits comes from the light microscopic examination of NR1-ir (Huntley *et al.*, 1994, 1997). We therefore investigated the cellular localization of NR1 and NR2A/B subunits of the NMDA receptor in the human cerebral cortex using immunocytochemical techniques in bioptic samples. To address this issue, we used antibodies that recognize many NR1 splice variants or the NR2A and B subunits (Petralia *et al.*, 1994a,b; see Discussion).

## Materials and Methods

### *Tissue Preparation*

Human cortical tissue came from the surgical specimens of five patients with brain tumors whose clinical data are summarized in Table 1. Three of the five tissue samples have been used also for another study (Conti *et al.*, 1998). Informed consent to the surgical procedure was obtained in all cases.

The cortical tissue used in this study was not located in the vicinity of the tumor: it was macroscopically normal cortical tissue included in 'tactical lobectomies' or that had to be resected in order to reach deep-seated tumors, and showed no signs of edema. None of the patients suffered from pre- or post-operative seizures. The approximate localization of the resected cortical samples is shown in Figure 1.

Tissue samples, all from the right hemisphere, were immediately immersed in a cold solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 2–3 h, and then transferred to a fresh solution of 4% PFA for 24–48 h at 4°C. Samples were cut into small blocks that were further postfixed for 24–72 h at 4°C in the same solution. They were cut on a Vibratome in either coronal or parasagittal planes into 30–35 μm thick sections, collected in groups of five, and stored at 4°C in phosphate-buffered saline (PBS) until processing. Of the five sections, one was stained for Nissl substance with 0.1% thionine and the remaining four were used for immunocytochemistry.

### *Immunocytochemistry*

#### *Antibodies*

The polyclonal antibodies against NR1 (AB1516/31697190) and NR2A/B (AB1548/90597149) used in the present study were purchased from Chemicon (Temecula, CA). Antibodies to NR1 were raised in rabbits against a 30 amino acid peptide corresponding to the C terminus of the rat NR1 subunit (Moriyoshi *et al.*, 1991), while antibodies to NR2A/B were raised against a 20 amino acid synthetic peptide conjugated to bovine serum albumin (BSA) with glutaraldehyde which corresponds to the C-terminal sequence of NR2A (Monyer *et al.*, 1992). Immunoblot analysis of human embryonic kidney cells (HEK-293) transfected with NMDA receptor subunits showed that the NR1 antibody is specific for this subunit and that it recognizes four NR1 splice variants (Petralia *et al.*, 1994a), whereas the NR2A/B antibody recognizes NR2A and NR2B and, to a lesser extent, NR2C and NR2D (Petralia *et al.*, 1994b). Further details on characterization and immunocytochemical application of these antibodies in the rat brain are reported in the original papers (Petralia *et al.*, 1994a,b). In the present material, method specificity was controlled by substituting the primary antibodies with PBS or normal sheep serum (NSS) and by preadsorbing the NR1 synthetic peptide conjugated to BSA with the working dilution of both NR1 and NR2A/B (50 μl of the conjugate in 1 ml of antibodies concentrated at 1.5 μl/ml) before immunocytochemical processing.

Monoclonal antibodies to glial fibrillary acidic protein (GFAP; Eng, 1971; Bignami and Dahl, 1973), an astrocytic marker, were purchased from Sigma (St Louis, MO; clone G-A-5, G-3893/1014–4800).

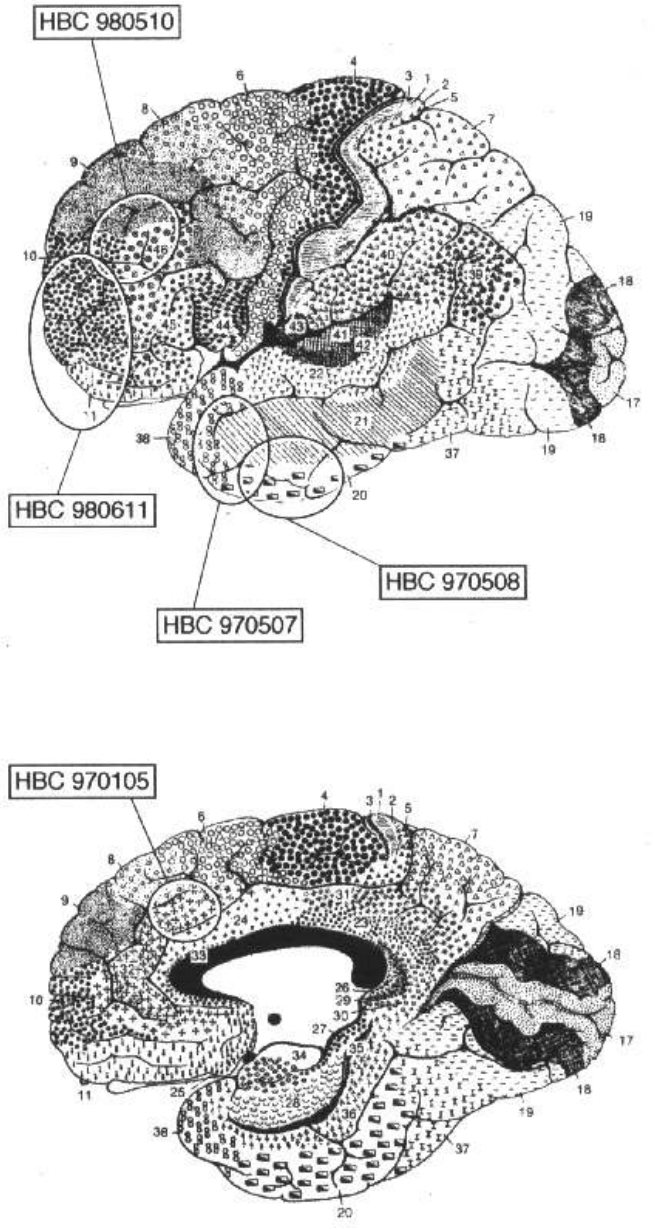



**Figure 1.** Diagram based on the surgeon's estimate of the cortical resection and on magnetic resonance imaging findings, showing the approximate localization of the cortical samples used in the present study superimposed on Brodmann's cyto-architectonic map of the human cerebral cortex. Note that whereas in Brodmann's maps the lateral view of the brain refers to the left hemisphere, all our samples were from the right hemisphere.

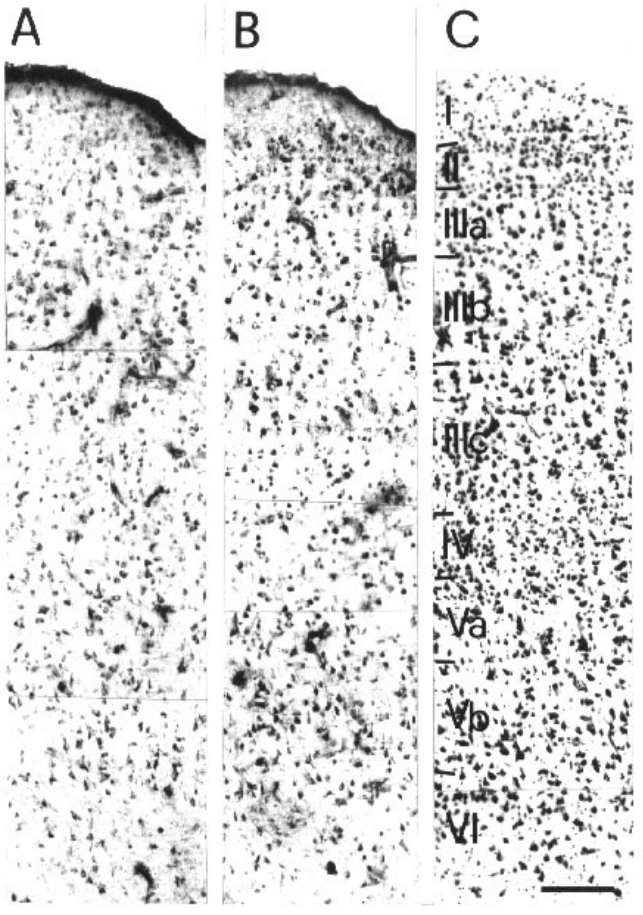



**Figure 2.** Distribution of NR1 (*A*) and NR2A/B (*B*) immunoreactivity in frontal cortex (area 46). (*C*) Adjacent section stained for Nissl substance. Roman numerals indicate cortical layers. Case no. HBC980510. Scale bar: 100 μm.

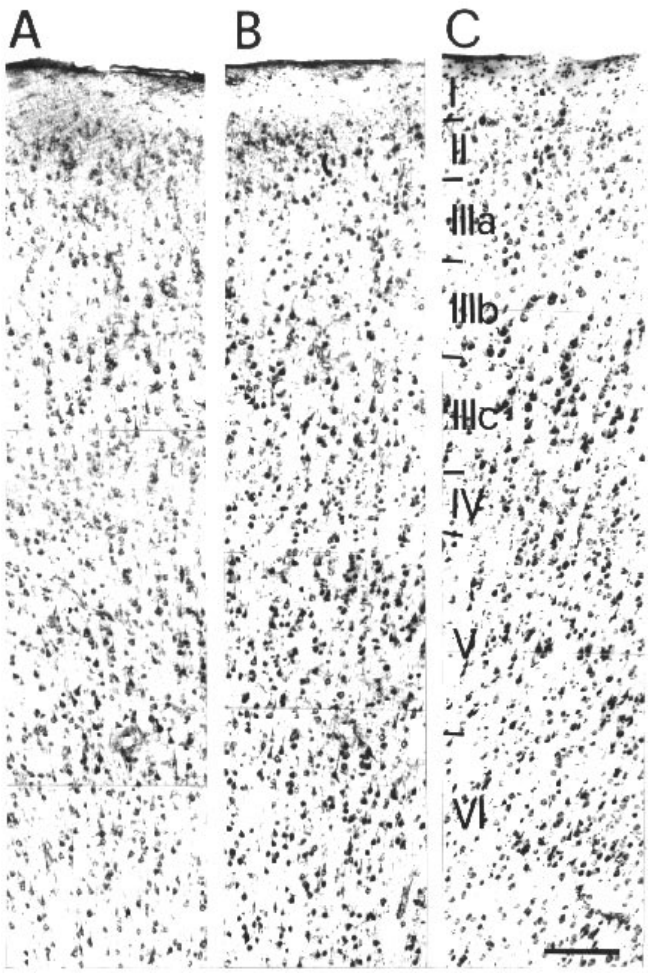



**Figure 3.** Distribution of NR1 (*A*) and NR2A/B (*B*) immunoreactivity in temporal cortex (area 21). (*C*) Adjacent section stained for Nissl substance. Roman numerals indicate cortical layers. Case no. HBC970508. Scale bar: 100 μm.

*Procedure*

Sections from human cortical blocks were pretreated with a solution of ethanol and hydrogen peroxidase in PB to remove endogenous peroxidase activity, washed free-floating in PBS and incubated in the following solutions: 1% normal goat serum (NGS; 1 h) containing 0.3% Triton X-100 (GFAP); 10% NSS only (NR1 and NR2A/B). Sections were then washed in PBS and incubated overnight in primary antiserum at dilutions of 1.5–2.0 μg/ml (NR1 and NR2A/B) and 1:1000 (GFAP) in PBS. Sections were rinsed, incubated in biotinylated anti-rabbit (NR1 and NR2A/B) or anti-mouse (GFAP) secondary antibodies diluted 1:100 (NR1 and NR2A/B) or 1:200 (GFAP) in PBS for 1–2 h at room temperature, rinsed in PBS, then incubated in avidin–biotin peroxidase complex (ABC; Vector, Burlingame, CA) (Hsu *et al.*, 1981) for 0.5 h, and rinsed in PBS. They were then incubated in 30–40 mg/100 ml 3–3′-diaminobenzidine (DAB; Sigma) with 0.02% $H_20_2$ in 0.05 M Tris buffer, pH 7.6, washed in PBS, mounted on gelatin-coated slides, air-dried, washed, dehydrated and finally coverslipped.

For electron microscopy, a mild ethanol pretreatment (10%, 25%, 10%; 5 min each) was applied before the immunocytochemical procedure. NR1 and NR2A/B antibodies were used at a concentration of 1.5–2.0 μg/ml and Triton X-100 was not used. After completion of the immunocytochemical procedure, sections were washed in PB, incubated in 2.5% glutaraldehyde (30 min), washed in PB and postfixed for 1 h in 1% $OsO_4$. After dehydration in ethanol and infiltration in Epon-Spurr resin, sections were flat-embedded between two Aclar (Sigma, 8F119)-coated coverslips. Small blocks, selected by light-microscopic inspection, were cut out, glued to blank-cured epoxy and sectioned with an ultramicrotome. Thin sections were lightly stained with lead citrate or left unstained and examined with a Philips CM10 electron microscope. Identification of neuronal and non-neuronal elements was performed using the criteria of Peters *et al.* (1991).

Cytoarchitectonic areas and boundaries were identified on thionine-stained adjacent sections according to Brodmann (1909), von Economo (1928), Ong and Garey (1990), Rajkowska and Goldman-Rakic (1995) and Vogt *et al.* (1995).

## Results

In the five cases studied in the present investigation, the sections adjacent to those used for the immunocytochemical detection of NR1 and NR2A/B were processed for the visualization of Nissl substance and GFAP. Examination of Nissl-stained sections showed that they were consistent with previous descriptions and devoid of any appreciable abnormality (Ong and Garey, 1990; Rajkowska and Goldman-Rakic, 1995; Vogt *et al.*, 1995; Marco *et al.*, 1996). The analysis of sections processed for GFAP evidenced abundant GFAP-positive cells and processes in layer I and in the white matter, but they were rare or absent in layers II–VI, in line with previous descriptions in normal tissue (Hansen *et al.*, 1987; Marco *et al.*, 1996; Perzel'ova *et al.*, 1997).

In areas 10, 46, 21 and 32, NR1 and NR2A/B ir were localized to neuronal cell bodies, to proximal portions of dendrites, to

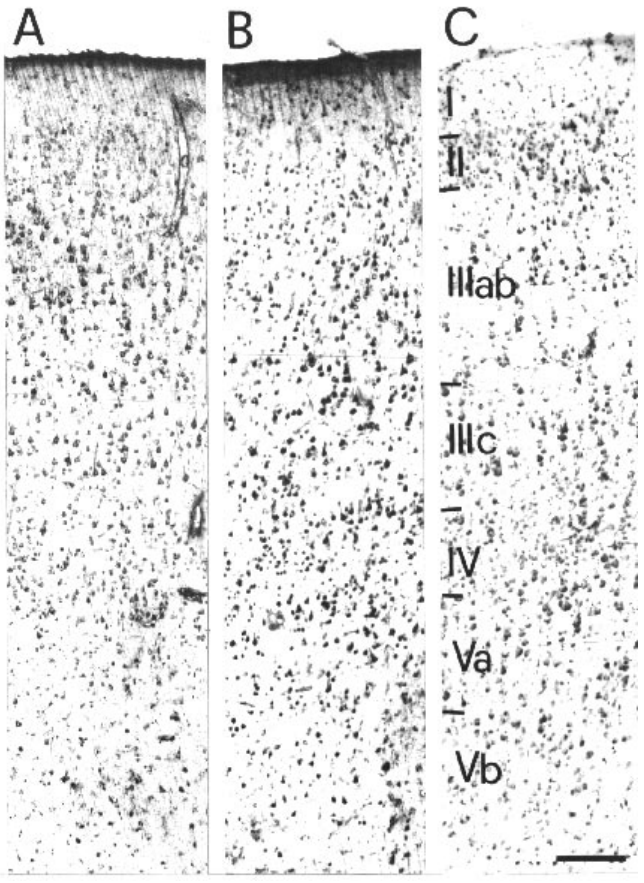



**Figure 4.** Distribution of NR1 (*A*) and NR2A/B (*B*) immunoreactivity in cingulofrontal transition cortex (area 32). (*C*) Adjacent section stained for Nissl substance. Roman numerals indicate cortical layers. Case no. HBC970507. Scale bar: 100 μm.

numerous small punctate structures, and to puncta and fragments of thin fibers within the neuropil (Figs 2–5). Similar patterns of immunoreactivity were observed in sections processed for the visualization of NR1 and NR2A/B (Figs 2–4). Light-microscopic analysis of NR1 and NR2A/B immunocytochemically stained sections revealed that in the cerebral cortex astrocytes, oligodendrocytes, microglial cells and other non-neuronal cells appeared always unlabeled.

In areas 10, 46, 21 and 32 NR1- and NR2A/B-positive neurons were in all cortical layers, though they appeared more numerous in layers II and III, while in infragranular layers they were more numerous in area 21 (Figs 2–4). The intense perikaryal labeling, continuous with an equally intense dendritic labeling, made it possible in most cases to distinguish pyramidal from nonpyramidal neurons (Conti *et al.*, 1987, 1992 for criteria). In all sections examined, NR1- and NR2A/B-positive neurons were both pyramidal and nonpyramidal (Fig. 5), though labeled pyramidal cells appeared more numerous than nonpyramidal ones.

Electron-microscopic studies showed that, with both antisera, the ultrastructural pattern of labeling was similar in all layers of the cortex. The electrondense reaction product of diaminobenzidine indicating NR1 and NR2A/B ir was present in neuronal cell bodies and processes, in axon terminals and in astrocytic profiles (Figs 6 and 7).

Neuronal labeling was found throughout the somatic and dendritic cytoplasm, but not in the nucleus (Figs 6*A*,*B*, 7*A*,*B*). Labeled perikarya showed a patchy distribution of reaction product, often associated with several cytoplasmic organelles (Figs 5*B*, 6*B*). In the neuropil, numerous dendrites of all sizes (Figs 6*F*, 7*D*, 8*B*) and dendritic spines of variable shape were intensely immunostained. Labeled dendritic spines received asymmetric synapses from unlabeled boutons with round, clear vesicles (Figs 6*D*,*F*, 7*E*). In addition, in all the samples examined, immunoreactivity was consistently found in the preterminal and terminal portions of axons (Figs 6*C*,*E-G*, 7*D*,*E*, 8*B*) and in a few myelinated axons (not shown). Labeled terminals showed mostly an asymmetric synaptic specialization (Figs 6*F*, 7*D*,*E*, 8*B*) and less frequently a symmetric one (asterisk in Fig. 6*E*) and they contacted either unlabeled dendritic spines or distal dendritic shafts.

Glial labeling was confined to distal astrocytic processes (Figs 6*F*,*G*, 7*C*,*F*, 8*C*), whereas astroglial cell bodies were occasionally weakly labeled. No reaction product was found in oligodendrocytes (not shown). Astrocytic processes were characterized by extremely irregular contours (Figs 6*F*,*G*, 7*F*) or by their perivascular location beneath the endothelial basal lamina of capillaries (Fig. 7*C*). Labeled astrocytic processes in the neuropil generally surrounded labeled (Fig. 6*F*) or unlabeled (Figs 6*F*, 7*E*, 8*C*) axonal terminals with asymmetric specialization, but were also present in areas not related with synapses.

In sections incubated in the antiNR1 serum preadsorbed with the NR1 peptide, no labeling was observed either at light- (not shown) or electron-microscopic (Fig. 8*A*) level, whereas in sections incubated in the antiNR2A/B serum preadsorbed with the NR1 peptide the pattern of immunolabeling was analogous to that observed in sections processed with the NR2A/B antibody at both the light- (not shown) and electron-microscopic level (Fig. 8*B*,*C*).

## Discussion

The present investigation of the distribution and cellular localization of the NR1 and NR2A/B subunits of the NMDA receptors in the human cerebral cortex confirmed the robust expression of this subtype of glutamate (Glu) receptors suggested by previous binding studies (Jansen *et al.*, 1989; Albin *et al.*, 1991) and revealed that: (i) the distribution of the NR2A/B subunits of the NMDA receptor is in all respects similar to that of NR1; (ii) dendritic spines express high levels of NR1 and NR2A/B subunits; (iii) both NR1 and NR2A/B subunits are localized also to excitatory and inhibitory axon terminals; and (iv) both NR1 and NR2A/B subunits are expressed by astrocytes. Thus, the effects of cortical NMDA activation in human cortex do not seem to depend exclusively on the opening of NMDA channels located at postsynaptic sites, and the localization of NMDA receptors appears to be similar in a variety of mammalian species.

### *Methodological Considerations*

This study was designed to investigate the distribution of NR1 and NR2A/B ir in normal samples of human cerebral cortex. Accordingly, we selected cortical tissue that was macroscopically normal in appearance and that was included in tactical lobectomies or resected to reach deep-seated tumors in patients who had never suffered from pre- or postoperative seizures. We also verified histologically that the sections adjacent to those used for the immunocytochemical visualization of NR1 and NR2A/B exhibited no obvious abnormalities in their architectonics or GFAP ir. These strict criteria of sample selection and the histological controls allow us to assume that the cortical tissue samples used in the present study were normal. Since receptor immunoreactivity in rapidly fixed biopsy specimens is

much stronger than in autoptic samples (e.g. Huntley *et al.*, 1997), our study seems to have been performed under optimal experimental conditions.

To investigate the distribution of NR1 and NR2A/B in the cerebral cortex, affinity-purified polyclonal antibodies raised against synthetic peptides corresponding to the predicted C termini of rat NR1 (Moriyoshi *et al.*, 1991) and NR2A (Monyer *et al.*, 1992; Nakanishi, 1992) were used. As reported in Materials and Methods, immunoblot studies showed that the NR1 antibody is specific for this subunit and that it recognizes four NR1 splice variants (Petralia *et al.*, 1994a), whereas the NR2A antibody recognizes NR2A and NR2B and, to a lesser extent, NR2C and NR2D (Petralia *et al.*, 1994b). Preadsorption tests performed on the tissue used in these studies showed that NR1 ir is totally abolished in sections processed with antibodies that had been incubated overnight with the conjugate peptide, and that NR2A/B ir is unaffected by preadsorbtion of the NR2A/B antibodies with the NR1 conjugate peptide, thus indicating that NR2A/B antibodies does not cross-react with NR1. Although the possibility of a cross-reaction of NR1 or NR2A/B antibodies with unidentified proteins cannot be excluded, these data suggest that the antigens we have localized in these tissue samples are the NR1 and NR2A/B subunits of the NMDA receptors.

The rationale for the choice of the NR1 antibody used here lies in the fact that it recognizes many of the splice variants demonstrated to date (Zukin and Bennett, 1995). Given that NR1 splice variants are expressed differentially both at the cellular and the subcellular level (Laurie and Seeburg, 1994; Sheng *et al.*, 1994; Standaert *et al.*, 1994; Johnson *et al.*, 1996), the recognition of several NR1 splice variants allows to achieve the labeling of most NR1 subunits and, therefore, of most, if not all, NMDA receptors. The NR2A antibody selected for this study recognizes both NR2A and NR2B subunits: this property is particularly useful for the analysis of NR2 expression in the cerebral cortex, where NR2A and NR2B are the only NR2 subunits expressed prominently (Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993; Watanabe *et al.*, 1993; Wenzel *et al.*, 1995).

Three methods can be used for the ultrastructural immunocytochemical localization of receptors, i.e. pre-embedding immunoperoxidase and pre- and postembedding immunogold (see Lujan *et al.*, 1996; Ottersen and Landsend, 1997, for recent reviews). Although the postembedding immunogold method is the procedure of choice to detect the synaptic localization of receptors and for the quantitative analysis of receptor density, its major drawbacks are its very low sensitivity and the need to use nonstandard fixation and embedding procedures (Nusser *et al.*, 1994; Baude *et al.*, 1995; Phend *et al.*, 1995; Propatiloff *et al.*, 1996). Conversely, pre-embedding immunoperoxidase is the most sensitive procedure (Ottersen and Landsend, 1997) and allows correlations to be made between light- and electron-microscopical findings. It is therefore the method of choice to obtain information on the regional distribution of labeling and to visualize receptors at nonsynaptic sites (e.g. in distal glial processes). For the purpose of the present study, therefore, pre-embedding immunoperoxidase is clearly the method of choice.

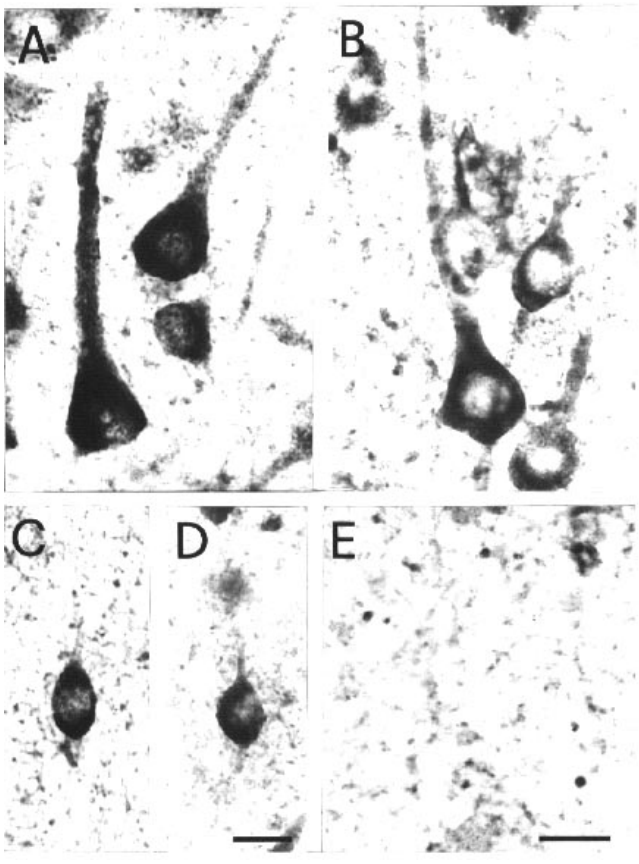
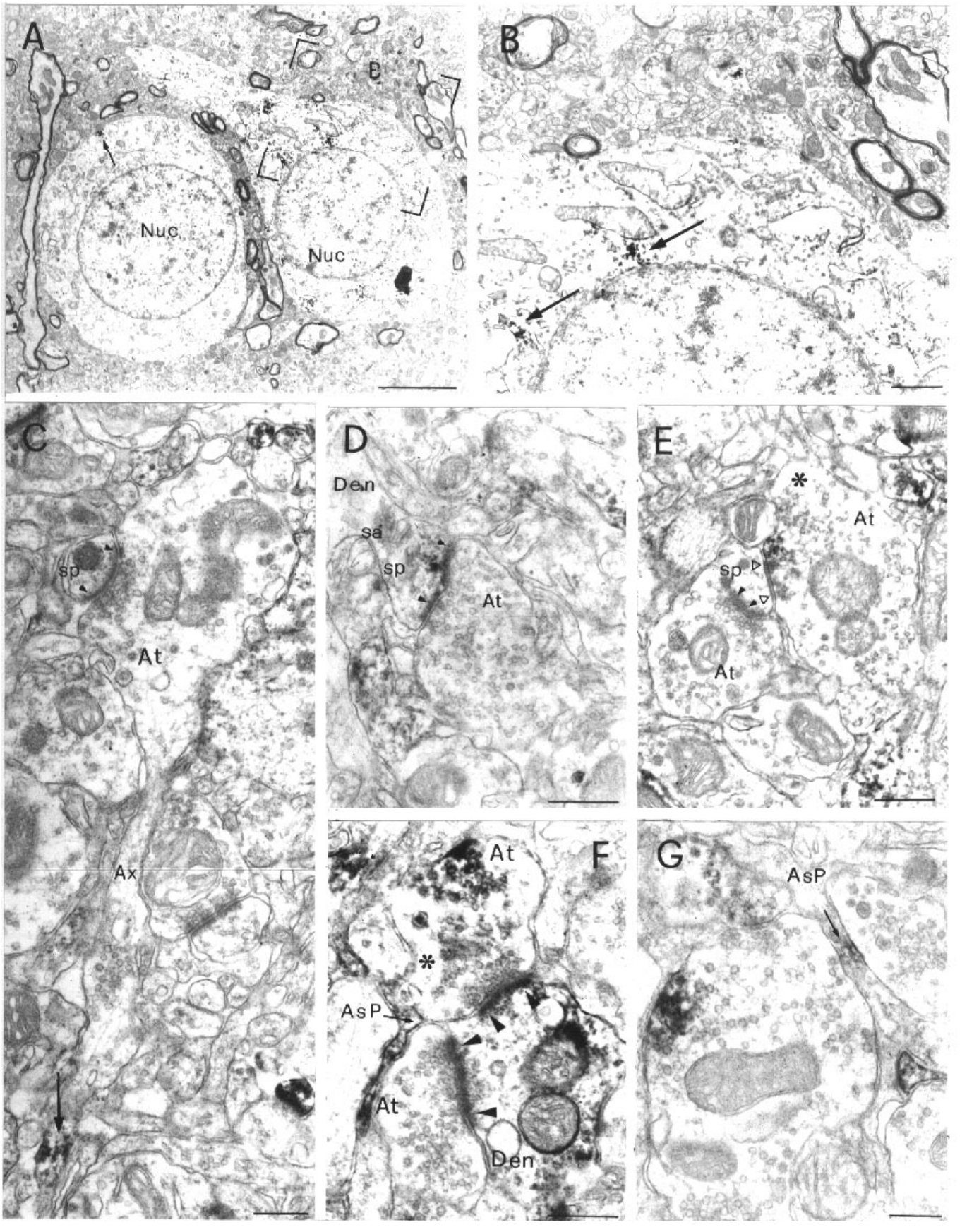



**Figure 5.** Morphology of NR1- and NR2A/B-positive neurons (*A–D*) and appearance of neuropilar staining (*E*) in area 21 of human cerebral cortex. (*A*) and (*B*) show some heavily stained pyramidal neurons in layers V (*A*) and II–III (*B*), that are NR1- (*A*) and NR2A/B (*B*)-positive, whereas (*C*) and (*D*) provide examples of NR1- (*C*) and NR2A/B (*D*)-positive nonpyramidal neurons. (*E*) shows NR1-positive puncta in the neuropil. Scale bar: 10 μm.

### *Distribution of NR1 and NR2A/B Subunits of the NMDA Receptor in the Human Cerebral Cortex*

Whereas the distribution of the NR1 subunit of the NMDA receptor has been analyzed at the light microscope in area 17 (Huntley *et al.*, 1994) and in the prefrontal and temporal association cortices (Huntley *et al.*, 1997), the distribution of the NR2A/B subunits has never been investigated in the human cerebral cortex. The results of the present light-microscopic analysis show that in areas 10, 46, 21 and 32 the distribution pattern of neurons expressing the NR2A and/or the NR2B subunit(s) is similar to that of neurons expressing NR1, and that the morphological features of NR1- and NR2A/B-positive neurons are also similar. These similarities in distribution are also evident

**Figure 6.** Ultrastructural localization of NR1 in human cortex. (*A*) Two neuronal perikarya with different degrees of ir. Arrow in (*A*) indicates a small clump of immunoreaction product. (*B*) Clumps of reaction product (arrows) scattered in the cytoplasm of the neuron framed in (*A*). (*C*) NR1-ir in the preterminal portion of an axon whose terminal forms an asymmetric synapse with an unlabeled dendritic spine. (*D*) an unlabeled axon terminal forming an asymmetric synapse (arrowheads) with a dendritic spine (note the spine apparatus). (*E*) A labeled axon terminal (asterisk) forms a symmetric synapse (open arrowheads) with an unlabeled dendritic spine that forms also an asymmetric synapse (arrowheads) with an unlabeled axon terminal. (*F*) A labeled (asterisk) and an unlabeled axon terminal forming asymmetric synapses (arrowheads) with a labeled dendrite. (*G*) A labeled distal astrocytic profile (arrow) is adjacent to a labeled (asterisk) and an unlabeled axon terminal. Abbreviations: At, axon terminal; Ax, axon; Den, dendrite; sp, spine; sa, spine apparatus; AsP, astrocytic process; Nuc, nucleus. (*A–G*) Lead citrate counterstaining. Case no. HBC980510. Scale bars: (*A*) 5 μm; (*B*,*D–F*) 1 μm; (*C*,*G*) 0.5 μm.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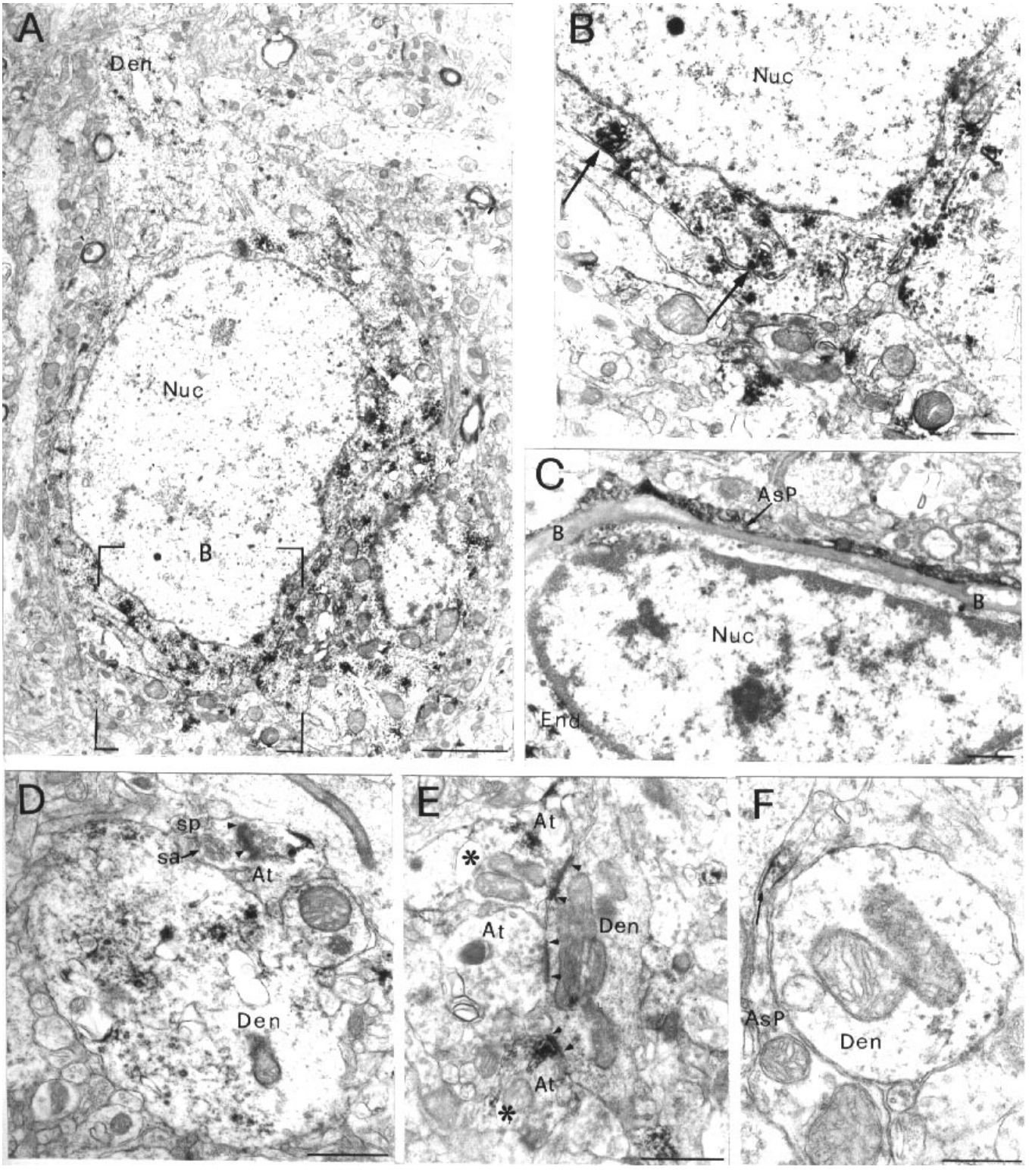


**Figure 7.** Ultrastructural localization of NR2A/B in human cortex. (*A*,*B*) Clumps of reaction product (arrows) in a neuronal cell body. (*C*) A labeled perivascular astrocytic profile (arrow) beneath the endothelium. (*D*) A labeled axon terminal forms an asymmetric synapse (arrowhead) with a spine stemming from a labeled dendrite. Note the spine apparatus (arrow). (*E*) Three axon terminals, two of which are labeled (asterisk), form asymmetric synaptic contacts (arrowheads) with an unlabeled dendrite. (*F*) A labeled distal astrocytic profile (arrow) adjacent to an unlabeled dendrite. Abbreviations: B, basal lamina; End, endothelial cell; for other abbreviations see legend to Figure 5. (*A–F*) Lead citrate counterstaining. Case no. HBC980510. Scale bars: (*A*) 5 μm; (*B–E*) 1 μm; (*F*) 0.5 μm.

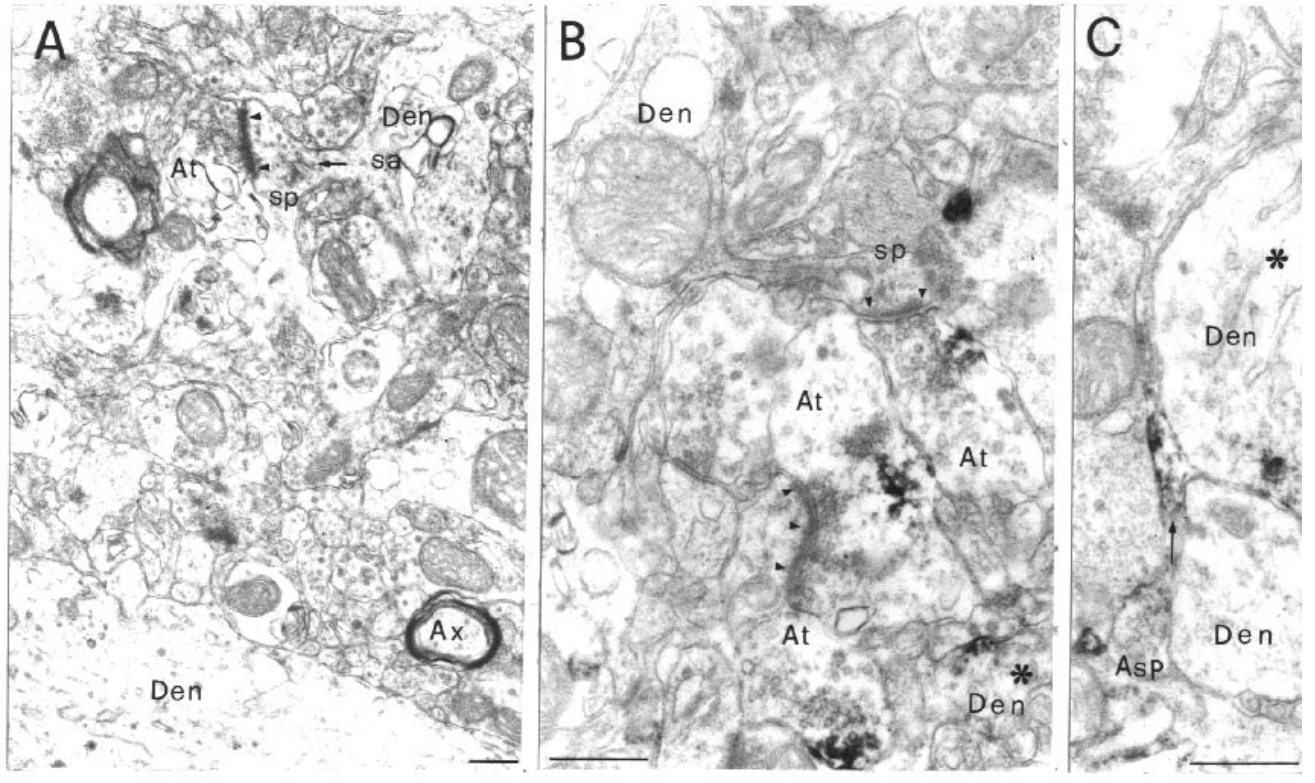


**Figure 8.** Preabsorption tests in human cerebral cortex. (*A*) A sample incubated in the anti-NR1 serum preasorbed with NR1 peptide does not show immunolabeling. (*B*,*C*) Samples incubated in the anti-NR2A/B serum preadsorbed with NR1 peptide show immunoreactivity in neuronal and glial processes. Case no. HBC980611. Scale bar: (*A*–*C*) 0.5 μm.

at the electron-microscopic level, as the cellular localization of NR2A/B is indistinguishable from that of NR1.

These data are in line with the results of previous *in situ* hybridization (Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992, 1994; Ishii *et al.*, 1993), immunocytochemical (Petralia *et al.*, 1994a,b; Conti *et al.*, 1997) and histoblot (Wenzel *et al.*, 1995) studies in rodent cerebral cortex which showed that the NR1 and NR2A and B subunits exhibit similar distribution patterns, and with the demonstration of triple subunit heteromeric NMDA receptors (NR1 + NR2A + NR2B) in cortical neurons (Sheng *et al.*, 1994; see also Conti *et al.*, 1997). Given that NR2C and NR2D are weakly expressed in rodent neocortex (Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992, 1994; Ishii *et al.*, 1993), these results have generated the notion that in the rodent neocortex most cortical NMDA receptors are formed by NR1 and NR2A and/or B subunits. Analysis of the expression patterns of mRNAs for NMDA receptor subunits in the human cerebral cortex (Akbarian *et al.*, 1996) supports this conclusion. Although the present results in human cerebral cortex do not address the stoichiometry of the human cortical NMDA receptors, they are nevertheless compatible with the notion that NMDA receptors in the cerebral cortex are formed preferentially by NR1 and NR2A and/or 2B subunits.

### *Neuronal Expression of NMDA Receptors in the Human Cerebral Cortex*

The present investigation shows that, in areas 10, 46, 21 and 32 of the human cerebral cortex, neurons expressing NMDA receptors are located in all layers, but are more numerous in layers II–III, followed by infragranular layers, and that in these layers they are in most cases pyramidal neurons. In previous studies, Huntley *et al.* (1994, 1997) have shown a similar distribution pattern in human area 17 and in prefrontal and temporal association cortices. This organizational feature thus appears to be consistent across different cortical fields of the human cerebral cortex. Interestingly, the same pattern has been described in several neocortical areas of adult rats (Aoki *et al.*, 1994; Conti *et al.*, 1994, 1997) and monkeys (Huntley *et al.*, 1994, 1997), which suggests that the distribution of NMDA receptors exhibits a striking interspecific similarity.

The electron-microscopic analysis performed in the present study has allowed us to garner information on the localization of NR1 and NR2A/B in the human cerebral cortex: both NR1 and NR2A/B exhibited a major localization to dendritic shafts and spines, and a minor localization to axon terminals. NR1 and NR2A/B immunoreactivities were concentrated in patches in the cytoplasm and dendrites, but the salient feature of NMDA receptors in human cortex is their localization to dendritic spines and shafts, which are in apposition to unstained axon terminals containing round vesicles. This localization pattern is identical to that observed in the neocortex of rats (Aoki *et al.*, 1994; Conti *et al.*, 1997) and monkeys (Huntley *et al.*, 1994, 1997), and is in line with a large amount of anatomical, electrophysiological and pharmacological data pointing to a preferential relationship between NMDA receptors and dendritic spines (see Conti *et al.*, 1997 for literature).

Ultrastructural studies showed that in the human cerebral cortex NMDA subunits R1 and R2A/B are localized to axon terminals forming both asymmetric and symmetric synapses. In previous investigations of the cellular localization of NMDA receptor subunits in the rat cerebral cortex, it has been shown

that NR1 is localized to axon terminals forming asymmetric (Aoki *et al.*, 1994; De Biasi *et al.*, 1996) and symmetric (De Biasi *et al.*, 1996) synapses, and that NR2A/B is expressed by axon terminals forming both symmetric and asymmetric synapses (Petralia *et al.*, 1994b; De Biasi *et al.*, 1996). In the monkey, rare NR1-positive axon terminals have been reported in primary cortices (Huntley *et al.*, 1994), but not in association cortices (Huntley *et al.*, 1997). Since presynaptic NMDA receptors have been reported also in the spinal cord (Liu *et al.*, 1994), locus coeruleus (Van Bockstaele and Colago, 1996) and hippocampus (Siegel *et al.*, 1994), presynaptic NMDA receptors appear to be a consistent, although minor, feature of brain organization. The identity of the synaptic transmitter released by NMDA-positive axon terminals has not been investigated directly in the present study; however, given that axon terminals forming asymmetric synapses are either Glu- or aspartate (Asp)-positive (DeFelipe *et al.*, 1988; Conti *et al.*, 1989), NMDA receptors in axon terminals forming asymmetric synapses are in all likelihood autoreceptors. The large majority of symmetric synapses in the neocortex are GABAergic (Kisvarday *et al.*, 1990), and double-labeling studies have shown that in rat neocortex some NMDA receptors are heteroreceptors on GABAergic axon terminals (De Biasi *et al.*, 1996); accordingly, the axon terminals forming symmetric synapses and expressing NMDA receptors observed in the present study are very likely to be GABAergic.

### *Glial Expression of NMDA Receptors in the Human Cerebral Cortex*

This study shows that in the human cerebral cortex, astrocytes express the NR1 and NR2A and/or NR2B subunits of the NMDA receptor. Given the specificity of the antibodies used in the present investigation, the NR1 and NR2A/B ir observed in astrocytic processes is unlikely to reflect cross-reactivity with other Glu receptors expressed by astrocytes (Blankenfeld, 1995; Gallo and Russell, 1995), though the possibility that the antibodies might have cross-reacted with other unidentified membrane proteins cannot be ruled out. The use of techniques for achieving good preservation of nervous tissue has allowed us to define the electron-microscopic features of astrocytes and astrocytic processes (Peters *et al.*, 1991), which can now be identified unambiguously (Peters *et al.*, 1991; Privat *et al.*, 1995; see also Minelli *et al.*, 1995, 1996 for recent data from our laboratory). The specificity of the antibodies used here and the conservative criteria used to identify astrocytic processes support the conclusion that some astrocytic processes in the human cerebral cortex do express NMDA receptors. Under the present experimental conditions, the electron microscopic approach was deemed preferable to that based on molecular astrocytic markers such as GFAP or S-100, since GFAP does not label all astrocytes (Peters *et al.*, 1991; Privat *et al.*, 1995) and since S-100 also labels some neurons and oligodendrocytes (Rickmann and Wolff, 1995; Richterlandsberg and Heinrich, 1995). In addition, the localization of NR1 and NR2A/B ir to distal astrocytic processes makes their visualization in light-microscopic double-labeling experiments with GFAP or S-100 extremely difficult and therefore unreliable. The present results are in agreement with our previous observations of GFAP-positive cells expressing mRNA for NR1 and of the presence of NR1 and NR2A/B ir distal astrocytic processes in the rat neocortex (Conti *et al.*, 1994, 1996), and emphasize the striking similarity in the localization of NMDA receptors between rodent and human cerebral cortex.

The preferential subcellular localization of NR1 and NR2A/B ir in astrocytic processes rather than in cell bodies presumably reflects the low cytoplasmic levels of a protein that is anterogradely transported, and suggests that they can monitor Glu release by neighboring axon terminals (DeFelipe *et al.*, 1988; Conti *et al.*, 1989) of thalamic and corticocortical origin, as well as by axon collaterals of cortical Gluergic neurons (Conti *et al.*, 1987, 1988; Kharazia and Weinberg, 1994), thereby mediating part of the neuron–glia signaling mechanisms that regulate gene expression and responses to changes in Glu levels (e.g. Stewart *et al.*, 1991; Herrera and Cuello, 1992; Uchihori and Puro, 1993). Astrocytic NMDA receptors may also participate in the mechanism(s) subserving activity-dependent cortical plasticity (Catalano *et al.*, 1997; Rao and Craig, 1997).

## Notes

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Address correspondence to Fiorenzo Conti, Istituto di Fisiologia Umana, Università di Ancona, Via Tronto, 10/A Torrette di Ancona, I-60020 Ancona, Italy. E-mail: f.conti@popcsi.unian.it.

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