Molecular Neurobiology

ISSN0893-7648/97/14(1): 1–18/$12.50

Neuronal and Glial Localization of NMDA Receptors in the Cerebral Cortex

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Abstract

The crucial role of glutamate receptors of the *N*-methyl-D-aspartate (NMDA) type in many fundamental cortical functions has been firmly established, as has its involvement in several neuropsychiatric diseases, but until recently, very little was known of the anatomical localization of NMDA receptors in the cerebral cortex of mammals. The recent application of molecular biological techniques to the study of NMDA receptors has allowed the production of specific tools, the use of which has much increased our understanding of the localization of NMDA receptors in the cerebral cortex. In particular, immunocytochemical studies on the distribution of cortical NMDA receptors have:

1. Demonstrated the preferential localization of NMDA receptors in dendritic spines, in line with previous work;
2. Disclosed a thus far unknown fraction of presynaptic NMDA receptors on both excitatory and inhibitory axon terminals; and
3. Shown that cortical astrocytes express NMDA receptors.

These studies indicate that the effects of cortical NMDA receptor activation are not caused exclusively by the opening of NMDA channels on neuronal postsynaptic membranes, as previously assumed, and that the activation of presynaptic and glial NMDA receptors can contribute significantly to these effects.

Index Entries: Glutamate; cerebral cortex; pyramidal neurons; nonpyramidal neurons; ionotropic receptors; autoreceptors; heteroreceptors; astrocytes; neuron-glia signaling.

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Introduction

The cerebral cortex is one of the brain regions in which glutamate (Glu) receptors of the *N*-methyl-D-aspartate (NMDA) type have a fundamental role, since they have been implicated in developmental processes, transmission of sensory information, synaptic plasticity, learning and memory, and neurotoxicity, as well as in a number of neurological and psychiatric diseases (Collingridge and Watkins, 1994; Conti and Hicks, 1996). Until a few years ago, information on cortical cells expressing NMDA receptors could be inferred exclusively from binding and electrophysiological studies (Monaghan et al., 1983, 1989; Greenamyre et al., 1985; Monaghan and Cotman, 1985; Thomson, 1986; Jones and Baughman, 1988; Maragos et al., 1988; Fox et al., 1989; Rosier et al., 1993; Currie et al., 1994; Thomson et al., 1996), which however provided little information on the cellular and subcellular localization of NMDA receptors.

NMDA receptors are formed by different subunits belonging to two classes: NMDAR1 (NR1) and NMDAR2 (NR2) (Monyer et al., 1992; Nakanishi, 1992; Cik et al., 1993; Wafford et al., 1993; Sheng et al., 1994; Mori and Mishina, 1995). The first subunit to be characterized, NR1, exhibits the basic features of the NMDA receptor when expressed in *Xenopus* oocytes (Moriyoshi et al., 1991), and can exist in several isoforms generated by alternative splicing (Zukin and Bennett, 1995). It has recently been shown that targeted disruption of the NR1 gene abolishes classical NMDA neuronal responses (Forrest et al., 1994), thus demonstrating that NR1 is an essential subunit of the NMDA receptor, and confirming previous suggestions based on expression studies of cDNA in heterologous cells and on the widespread distribution of NR1 mRNA in the central nervous system (Nakanishi, 1992). The second class of NMDA receptor subunits, NR2, 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). No splice variants have been reported for NR2A, B, and C, whereas NR2D exists in two forms, NR2D1 and NR2D2 (Ishii et al., 1993). Electrophysiological experiments indicate 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 *in situ* hybridization shows that they are differentially expressed in the brain (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993) and during development (Monyer et al., 1992; Farrant et al., 1994; Sheng et al., 1994). These findings have generated the notion that NR2 subunits serve a modulatory role. Indeed, it has been shown that the combination of NR1 with different NR2 subunits modifies both electrophysiological and pharmacological responses (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; Molinoff et al., 1994).

Advances in the knowledge of the molecular biology of NMDA receptors have made available, among others, potent and specific tools for studying the localization of these receptors at the cellular level. Here, we briefly review the results of a series of recent anatomical investigations on the localization of NMDA receptors in the cerebral cortex of adult mammals.*

*Recently, a member of a novel class of rat ionotropic Glu receptor family has been cloned and termed X-1 (Ciabarra et al., 1995) or NMDAR-L (Sucher et al., 1995). This subunit, which exhibits about 27% identity to NMDA subunits, does not demonstrate agonist-activated currents in functional expression studies, but produces inhibition of responses when coexpressed with NMDA subunits, thus suggesting that it specifically interacts with NMDA receptors and determines changes in their functional state. However, given the current uncertainty of its functional role and the low intensity of hybridization in the cortex of adult animals (although not during development), this subunit is not considered in the present article.

Neuronal Expression of NMDA Receptor Subunits

NR1

In sections of rat and monkey neocortex incubated with the NR1 antisense RNA probe, specific hybridization signal is present over a large number of neurons, especially in layers II–III and V–VI (Fig. 1A). Evaluation of cortical neurons expressing NR1 mRNA reveals that in the rat cerebral cortex, about 80% of all neurons are associated with silver grains (Conti et al., 1994a). With the exception of layer I, in which only 6.5% of neurons are labeled, the proportion of labeled neurons in the different layers is fairly homogeneous: 85% in layers II and III, 80% in layer IV and V, and 88% in layer VI. Labeled neurons, however, can be differentiated on the basis of the number of silver grains they are associated with (Conti et al., 1994a; Conti and Minelli, 1996), and it has been noted that neurons expressing high levels of signal are particularly concentrated in layers II–III and V. Although the possibility that post-translational mechanisms regulate the amount of subunit produced in a given cortical neuron cannot be excluded, the observation that different groups of neurons express variable numbers of NR1 subunits strongly suggests that they also express a variable number of functional receptors (Conti et al., 1994a; Conti and Minelli, 1996).

Light microscopic examination of sections of mammalian neocortex stained with anti-NR1 antibodies shows that NR1 immunoreactivity (IR) is localized to neuronal cell bodies, proximal portions of dendrites and (in some cases) secondary dendrites, and to small punctate structures and fragments of thin fibers within the neuropil (Figs. 2A; 3A,D,E; *see also* Aoki et al., 1994; Huntley et al., 1994, 1996; Conti and Minelli, 1996). In rat neocortex, NR1+ neurons are about 70% of cortical neurons; they are in all cortical layers (Fig. 2A), but more numerous in layers II (76%) and III (77%), followed by layers V (71%), VI (69%), IV (63%), and I (39%).** The slight difference between this estimate and that from *in situ* hybridization material is accounted for by the different sensitivity of the techniques employed (Benson et al., 1989, 1991; Jones, 1993). The intense perikaryal labeling continuous with an equally intense

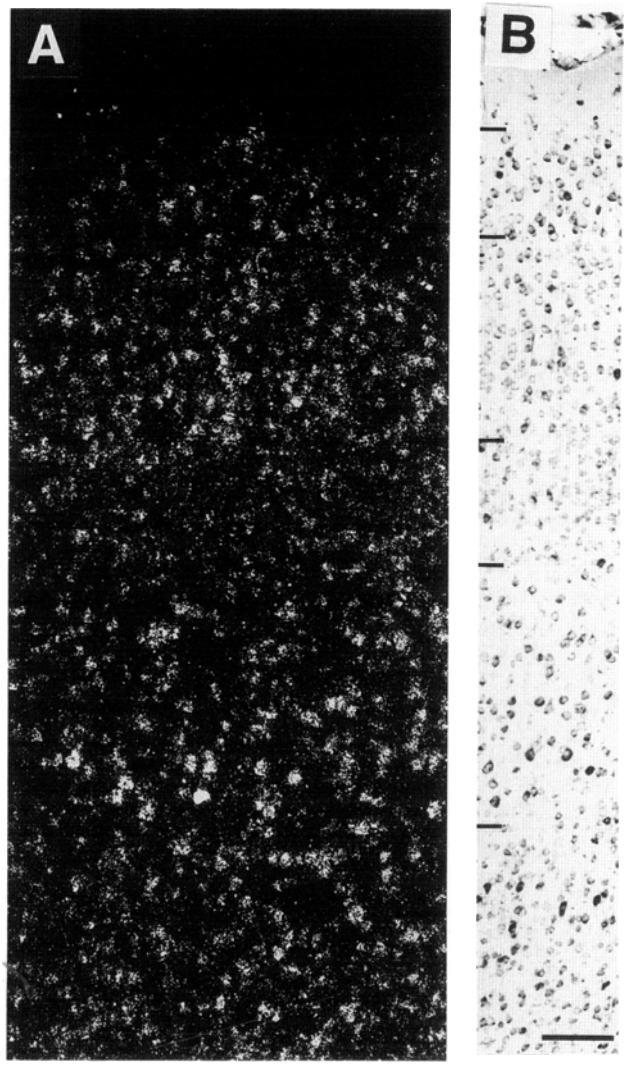

Fig. 1. **(A)** Distribution of NR1 transcripts in the first somatic sensory cortex of adult rats. **(B)** Toluidine blue staining of the same section. Scale bar: 70 μm. Modified from Conti et al. (1994a).

**Semiquantitative data on the percentage of NR1 and NR2A/B immunoreactive neurons and on the ratio of labeled pyramidal to nonpyramidal neurons were collected as described in Conti et al. (1987, 1992).

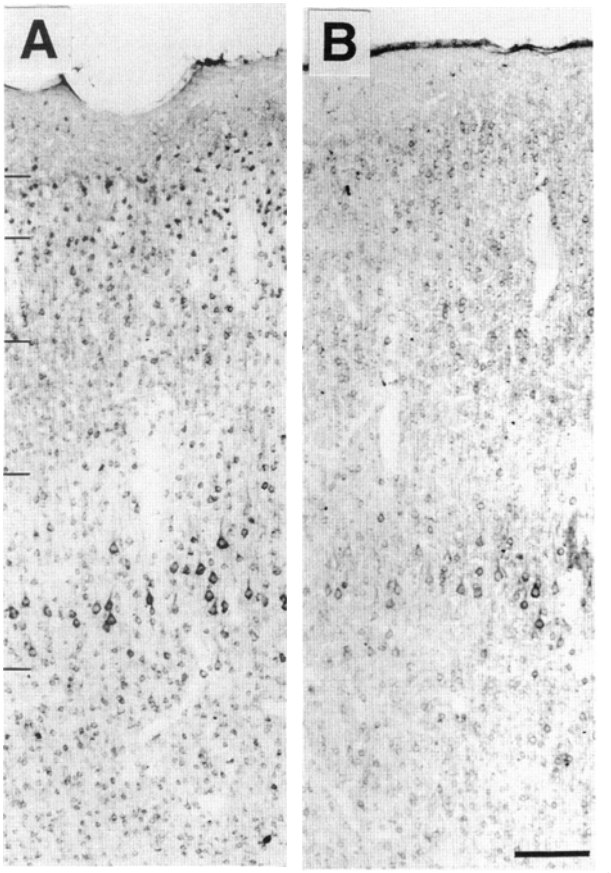

Fig. 2. Distribution of NR1 **(A)** and NR2A/B **(B)** IR in the SI cortex. Bar: 200 μm. Unpublished material of M. Melone, A. Minelli, S. DeBiasi, and F. Conti.

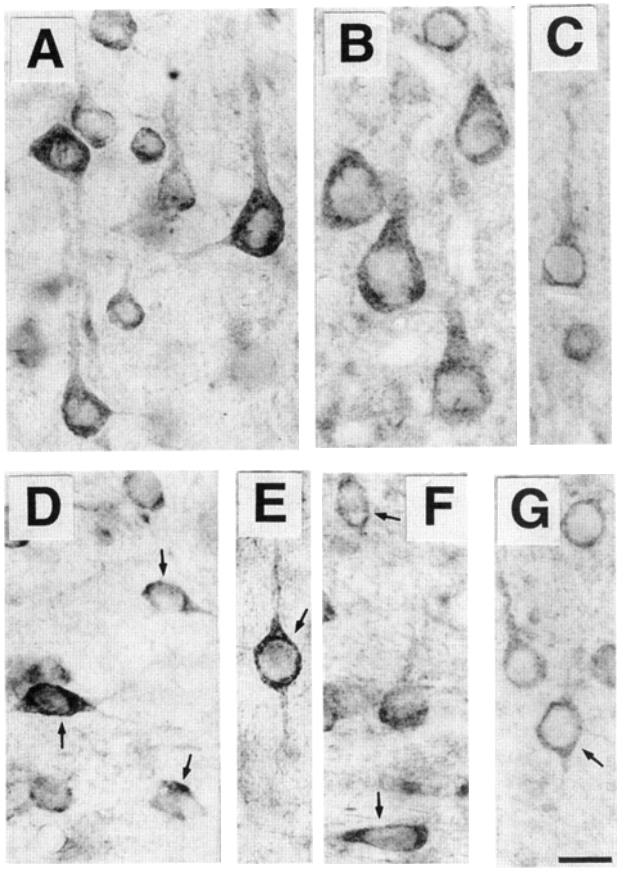

Fig. 3. Morphology of NR1 and NR2A/B+ neurons. **(A–C)** Pyramidal neurons expressing NR1 (A) and NR2A/B **(B,C)** immunoreactivity. **(D–G)** Nonpyramidal neurons (arrows) expressing NR1 (D,E) and NR2A/B (F,G) immunoreactivity. Bar: 15 μm. From Conti et al. (1996).

dendritic labeling makes it possible, in most cases, to distinguish between pyramidal and nonpyramidal neurons (Conti et al., 1987, 1992 for criteria). In rats, monkeys, and humans, NR1+ neurons are both pyramidal and nonpyramidal (Fig. 3A,D,E; Aoki et al., 1994; Huntley et al., 1994, 1996; Conti and Minelli, 1996), although (at least in rats) labeled pyramidal cells are more numerous than labeled nonpyramidal neurons (70 vs 30%, respectively; Conti and Minelli, 1996).

Electron microscopic analysis of NR1 IR in the cerebral cortex of rats and monkeys shows that NR1 IR is in the cytoplasm of neuronal cell bodies and dendrites, where it is concentrated in patches associated with microtubules, rough endoplasmic reticulum, Golgi apparatus, and nuclear envelope (Fig. 4B; *see also* Aoki et al., 1994; Petralia et al., 1994a; Huntley et al., 1994). In the neuropil, the most typical feature of NR1 IR is the staining of postsynaptic densities in dendritic spines and shafts, which are in apposition to unstained presynaptic terminals filled with round or pleomorphic vesicles (Fig. 4C; *see also* Aoki et al., 1994; Petralia et al., 1994a; Huntley et al., 1994, 1996). Some NR1+ axosomatic and axoaxonic asymmetric synapses have also been reported (Kharazia et al., 1996; *see also* Aoki et al., 1994). In all layers of both

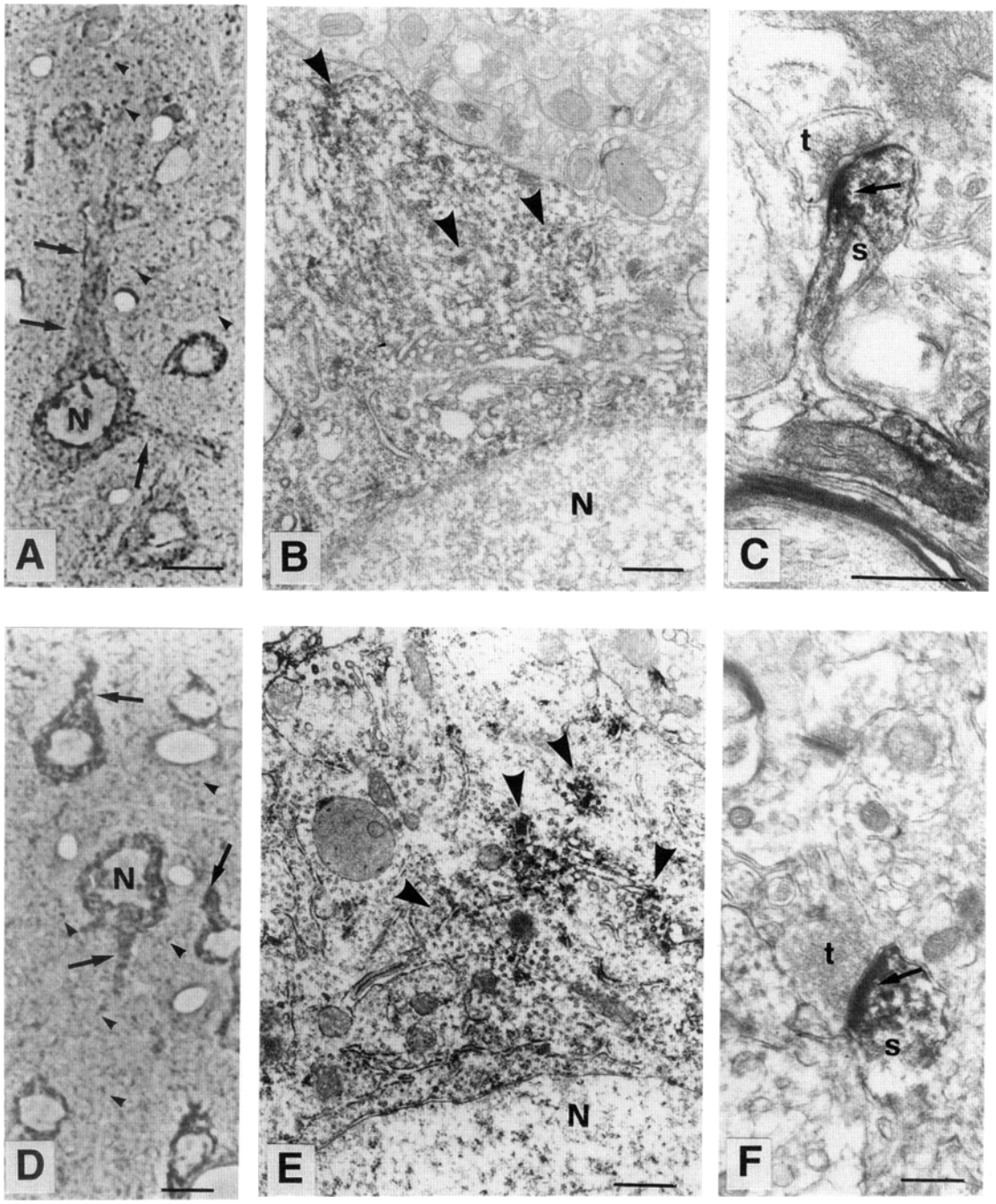

Fig. 4. NR1 and NR2A/B IR in neuronal cell bodies and in their processes. **(A,D)** Unstained semithin sections showing labeling for NR1 (A) and NR2A/B (D) in neuronal perikarya and major dendrites (arrows), but not in the nucleus (N). Immunoreactive puncta (arrowheads) are scattered throughout the neuropil. Phase-contrast microscopy. Scale bar: 10 μm. **(B,E)** Electron micrographs showing labeling for NR1 (B) and NR2A/B (E) in neuronal cell bodies. Clumps of reaction product (arrowheads) are scattered in the cytoplasm; the nucleus is unlabeled. Lead citrate counterstaining. Scale bars: 0.5 μm. **(C,F)** Electron micrograph showing labeling for NR1 (C) and NR2A/B (F) in dendritic spines contacted by unlabeled axon terminals (t) with round vesicles and asymmetric specialization. Arrows point to aggregation of reaction product at postsynaptic sites. Scale bars: 0.5 μm.

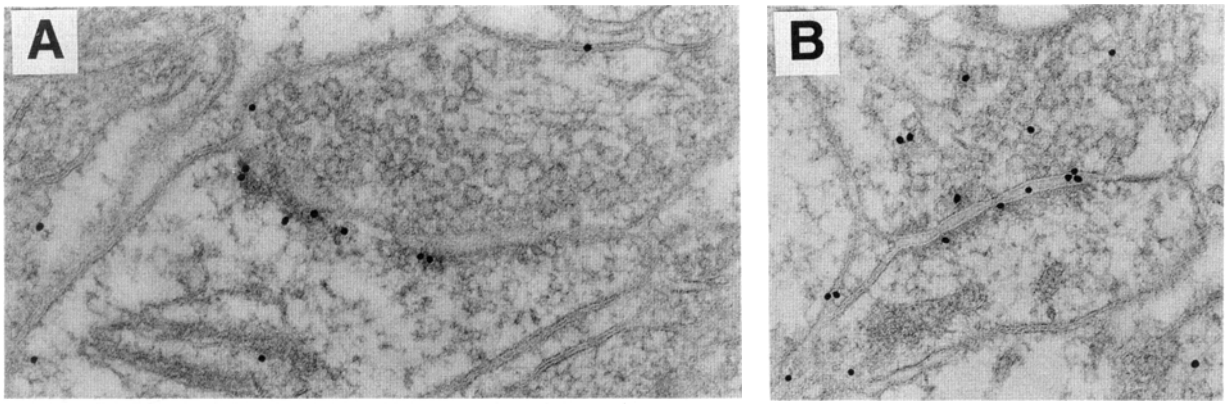

Fig. 5. NR1 IR in axospinous synapses of the rat somatic sensory cortex (layer III) visualized by an immunogold technique (Wenthold's antibody). **(A)** Gold particles coding for NR1 are concentrated at the active zone. **(B)** An NR1+ axon terminal forming an asymmetric synapse with a NR1+ spine. × 75,000. Courtesy of V. Kharazia.

rat and monkey cortex, numerous asymmetric synapses are NR1+, but they are by far more numerous in supragranular than in infragranular layers (Aoki et al., 1994; Huntley et al., 1994). Immunogold studies confirm the association between NR1 IR and postsynaptic densities of asymmetric synapses, and show that NR1 IR is often, though not always, concentrated at the active zone (Kharazia et al., 1996; Fig. 5; *see also* Aoki et al., 1994).

Ultrastructural studies show that in the rodent and monkey cerebral cortex, some axon terminals exhibit NR1 IR (Aoki et al., 1994; Huntley et al., 1994; DeBiasi et al., 1996; Johnson et al., 1996; Weinberg et al., personal communication; but *see* Huntley et al., 1996). Aoki et al. (1994) report that a substantial portion (16%) of NR1 IR profiles in the adult neocortex are axon terminals containing clusters of reaction product over synaptic vesicles and forming asymmetric synapses with dendritic shafts and spines, either labeled or unlabeled. In our studies, we have observed that NR1 IR is consistently present in a few myelinated fibers and in axon terminals (Fig. 6A–D; DeBiasi et al., 1996; *see also* Fig. 5B). Reaction product within axon terminals is diffuse, surrounding vesicles and mitochondria, but it never extends to the synaptic cleft (DeBiasi et al., 1996). Labeled terminals are mostly of small size and make either asymmetric or symmetric synapses on unlabeled neuronal profiles, mainly on dendritic spines and occasionally on dendritic shafts (Fig. 6, A–D). Combining pre- and postembedding immunocytochemistry, we have observed that all NR1+ axon terminals with symmetric specialization are selectively enriched in gold particles coding for GABA (Fig. 6D; DeBiasi et al., 1996).

NR2

NR2A and B are highly expressed in the cerebral cortex of rats and mice, whereas NR2C and D exibit a very low intensity of signal (*in situ* hybridization: Monyer et al., 1992, 1994; Ishii et al., 1993; Watanabe et al., 1993; histo-blot: Wenzel et al., 1995). Cells expressing NR2A or B subunits do not display clear regional differences, and are numerous in layers II–III and V–VI (Fig. 7A,B).

Light microscopic studies of sections of cerebral cortex stained with antibodies recognizing both NR2A and NR2B (Petralia et al., 1994b) show that IR is localized to the same structures that are NR1+ (neuronal cell bodies, proximal portions of dendrites, and neuropilar puncta and fragments of thin fibers) (Fig. 2B; *see also* Petralia et al., 1994b; Conti and Minelli, 1996). In rat cerebral cortex, NR2A/B+ neurons

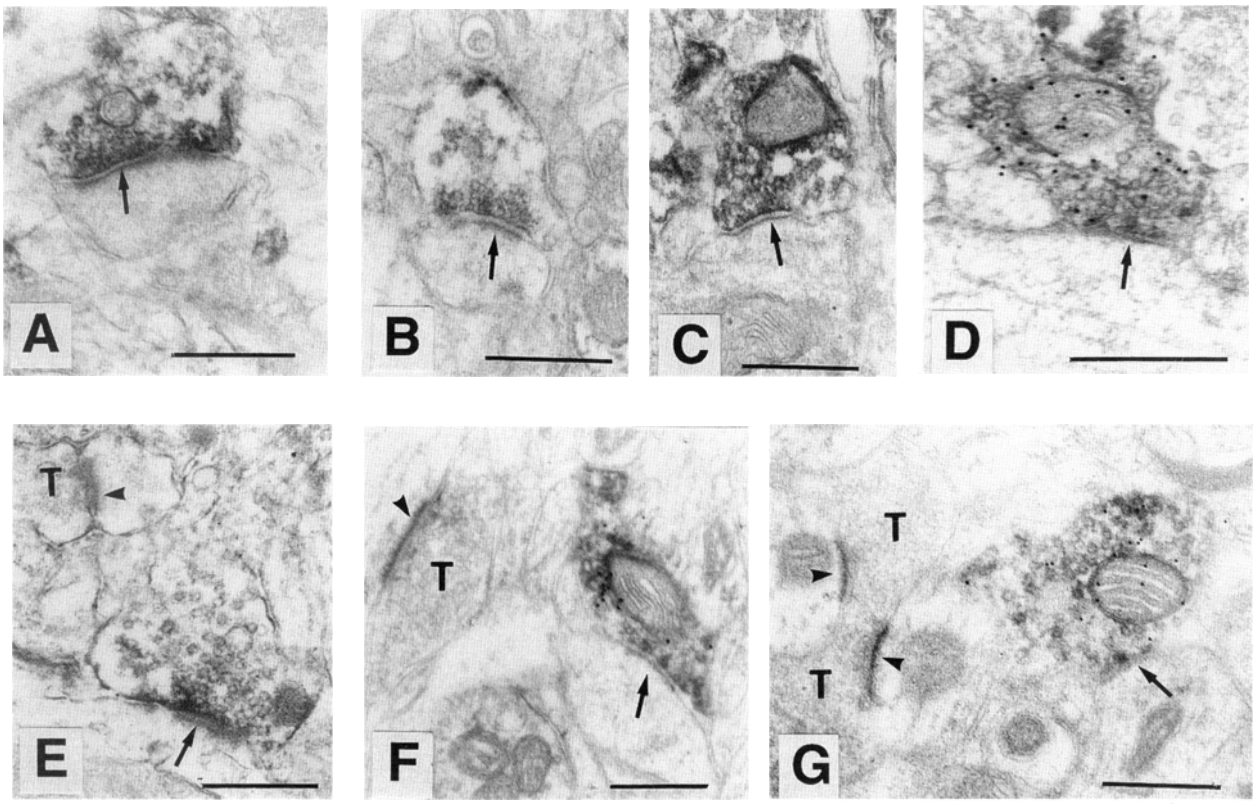

Fig. 6. NR1 **(A–D)** and NR2A/B+ **(E–G)** axon terminals. (A and B): NR1+ terminals forming asymmetric synapses (arrows) on unlabeled dendritic spines. (C and D): NR1+ terminals forming symmetric synapses (arrows) on unlabeled dendrites. The terminal in (D) is also labeled by gold particles coding for GABA. (E) NR2A/B+ terminal making asymmetric synapse (arrow) on an unlabeled dendrite. An adjacent terminal (T) also making an asymmetric synapse (arrowhead) is unlabeled. (F and G): NR2A/B+ terminals making symmetric synapses (arrows) on unlabeled dendrites are also labeled by gold particles coding for GABA. Adjacent terminals (T) making asymmetric synapses (arrowheads) are unlabeled. Bars: 0.5 μm. From DeBiasi et al. (1996).

are about 50% of all neurons; they are in all cortical layers (Fig. 2B), although they exhibit a differential laminar distribution. In all cases, the percentage of labeled neurons is highest in layers VI and III, followed by layers II and V (Conti and Minelli, 1996). This estimate seems to suggest that more neurons express NR1 than NR2. It is conceivable that the difference between the percentage of neurons expressing NR1 and of those expressing NR2A/B can be accounted for by neurons expressing NR2C and D. However, the lack of quantitative data on the expression of NR2C and D does not allow conclusions to be drawn, since semiquantitative analysis of material processed for *in situ* hybridization histochemistry has revealed that a number of neurons express very low levels of mRNA even in cases in which both film images and sections coated with photographic emulsion seem to indicate a virtual lack of expression (*see*, for instance, the data of Conti et al., 1994b on the expression of GluR4). NR2A/B+ neurons are both pyramidal and nonpyramidal (Fig. 3B,C,F,G; Conti and Minelli, 1996), but pyramidal cells are more numerous than nonpyramidal neurons (70 vs 30%, respectively; Conti and Minelli, 1996).

At the ultrastructural level, the pattern of NR2A/B IR in the rat cerebral cortex is in all respects similar to that of NR1 (Figs. 4 and 6). Reaction product is present throughout the cytoplasm of neuronal cell bodies and their

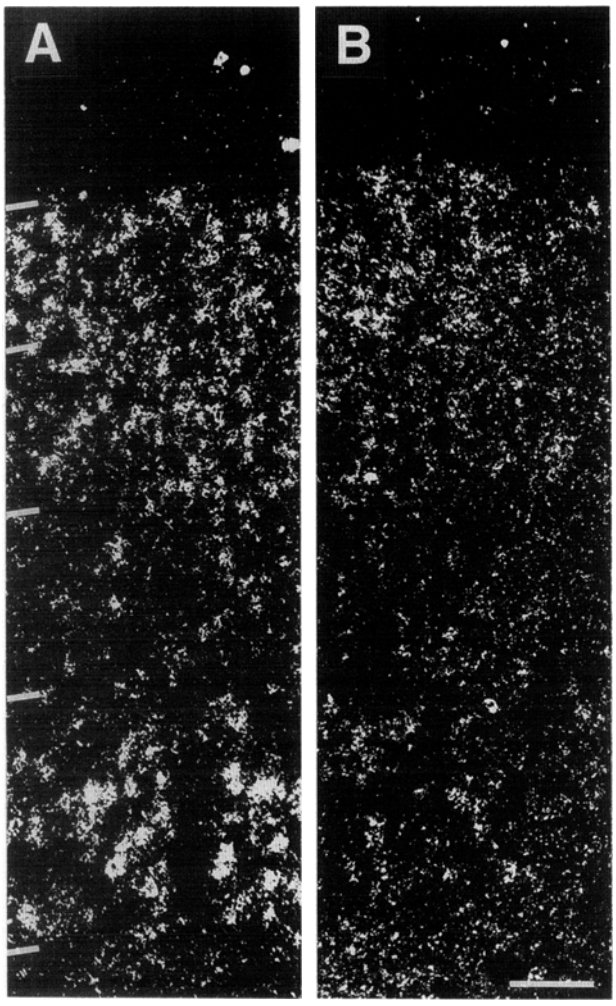

Fig. 7. Distribution of NR2A **(A)** and NR2B **(B)** transcripts in the first somatic sensory cortex of adult rats. ^{35}S-UTP-labeled mRNA probes obtained from a 917-bp fragment (3318–4235 of full-length rat NR2A cDNA) subcloned into *Eco*RV restriction site of pSKII(+) Bluescript vector, and from a 748-bp segment (3572–4320 of full-length rat NR2B cDNA) subcloned into *Apa*I restriction site of pKSII(+) Bluescript vector. Scale bar: 70 μm. Unpublished material of A. Minelli, N. C. Brecha, R. H. Edwards, and F. Conti.

processes, but not in the nucleus (Fig. 4E). In the neuropil, a considerable number of dendrites of all sizes and of dendritic spines of variable shape are intensely stained (Fig. 4F). Reaction product in dendritic spines is particularly evident in areas postsynaptic to small, unlabeled boutons with round clear vesicles and asymmetric specialization. As for NR1 immunolabeling, the synaptic cleft is always devoid of NR2A/B staining. NR2A/B IR is also present in axon terminals forming either symmetric or asymmetric synapses (Fig. 6E,F); the former are selectively enriched in GABA (Fig. 6F,G; DeBiasi et al., 1996).

NR1-NR2 Coexpression

The notion that native NMDA receptors result from the assembly of different subunits derives from data collected in vitro (Monyer et al., 1994) and in vivo only by coimmunoprecipitation (Sheng et al., 1994), which does not provide information on the cellular localization of the receptor subunits. We have studied the degree of NR1 and NR2A/B coexpression by using an immunocytochemical technique that allows reliable visualization of two antigens in the same neuron (Kosaka et al., 1985a,b). The large majority of NR1+ cells are also NR2A/B+; in some cases, however, cells are NR1+ but NR2A/B-, whereas cells that are NR2A/B+ and NR1- are rare (Fig. 8). Although definite evidence is not available, neurons expressing NR1, but not NR2A/B probably express NR2C and/or D. The presence of rare neurons that express NR2A/B, but not NR1, is presumably because NR1 antibodies recognize only four of the seven splice variants reported to date, and/or to the presence of below-threshold levels of NR1 IR.

Glial Expression of NMDA Receptors

Glial expression of NMDA receptors has been suggested in earlier *in situ* hybridization studies by the detection of hybridization signal over cells having the size and tintorial features of glial cells (Watanabe et al., 1993; Conti et al., 1994a; Laurie and Seeburg, 1994). Subsequently, double-labeling studies with *in situ* hybridization histochemistry and immunocy-

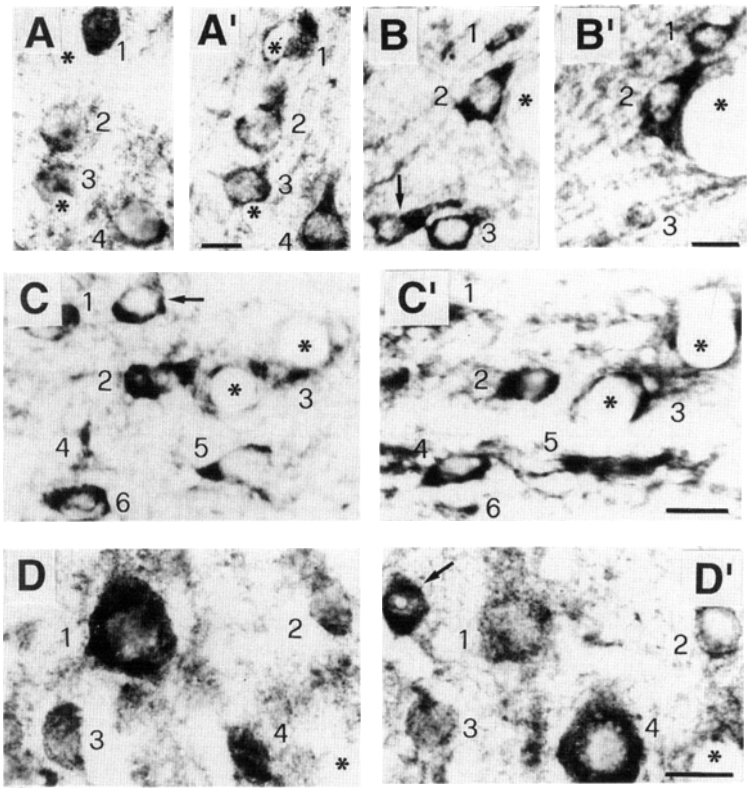

Fig. 8. Paired photomicrographs from confronting planes of two consecutive sections processed with NR1 (A–D) and NR2A/B (A'–D') antibodies. Asterisks indicate profiles of blood vessels. In A–A', all neurons (1–4) show colocalization of NR1 and NR2A/B. In B–B' and C–C', neurons 1–3 and 1–8 are positive to both antibodies, whereas neurons indicated by arrows express only NR1. In D–D', neurons 1-4 are positive to both antibodies, whereas the neuron indicated by the arrow expresses only NR2A/B. Note that some perikarya were cut near the center (A_{1-4}, B_2, C_4, and $D_{1,2}$), whereas others were cut through their peripheral portions (B_3, $C_{3,6,8}$, D_4). Bars: 15 μm for A–A'–C–C', 30 μm for D–D'. Unpublished material of M. Melone, A. Minelli, S. DeBiasi, and F. Conti.

tochemistry with antibodies to glial fibrillary acidic protein (GFAP) showed that in the rat cerebral cortex, 96% of GFAP+ cells do not express NR1 mRNA (Conti et al., 1994a). However, 4% of the GFAP+ cells express low levels of NR1 mRNA, thus showing for the first time that a small proportion of cortical astrocytes express NR1 (Conti et al., 1994a).

Compelling evidence for astrocytic expression of NMDA receptor subunits, however, has been provided only recently in immunocytochemical studies using specific antipeptide antibodies. As reported above, light microscopic analysis of NR1– and NR2A/B– immunocytochemically stained sections does not reveal the presence of NR1 or NR2A/B IRs in astrocytes, oligodendrocytes, microglial cells, and other nonneuronal cells. At the electron microscope, however, both NR1 (Aoki et al., 1994; Conti et al., 1996) and NR2A/B (Conti et al., 1996) IRs are detectable in several astrocytic processes (Fig. 9A–G), whereas astroglial cell bodies are only occasionally labeled. Labeled astrocytic processes are scattered in the neuropil, surrounding unlabeled axonal terminals with asymmetric or symmetric specialization and form end feet expansions beneath the endothelial basal lamina of capillaries (Fig. 9; Conti et al., 1996).

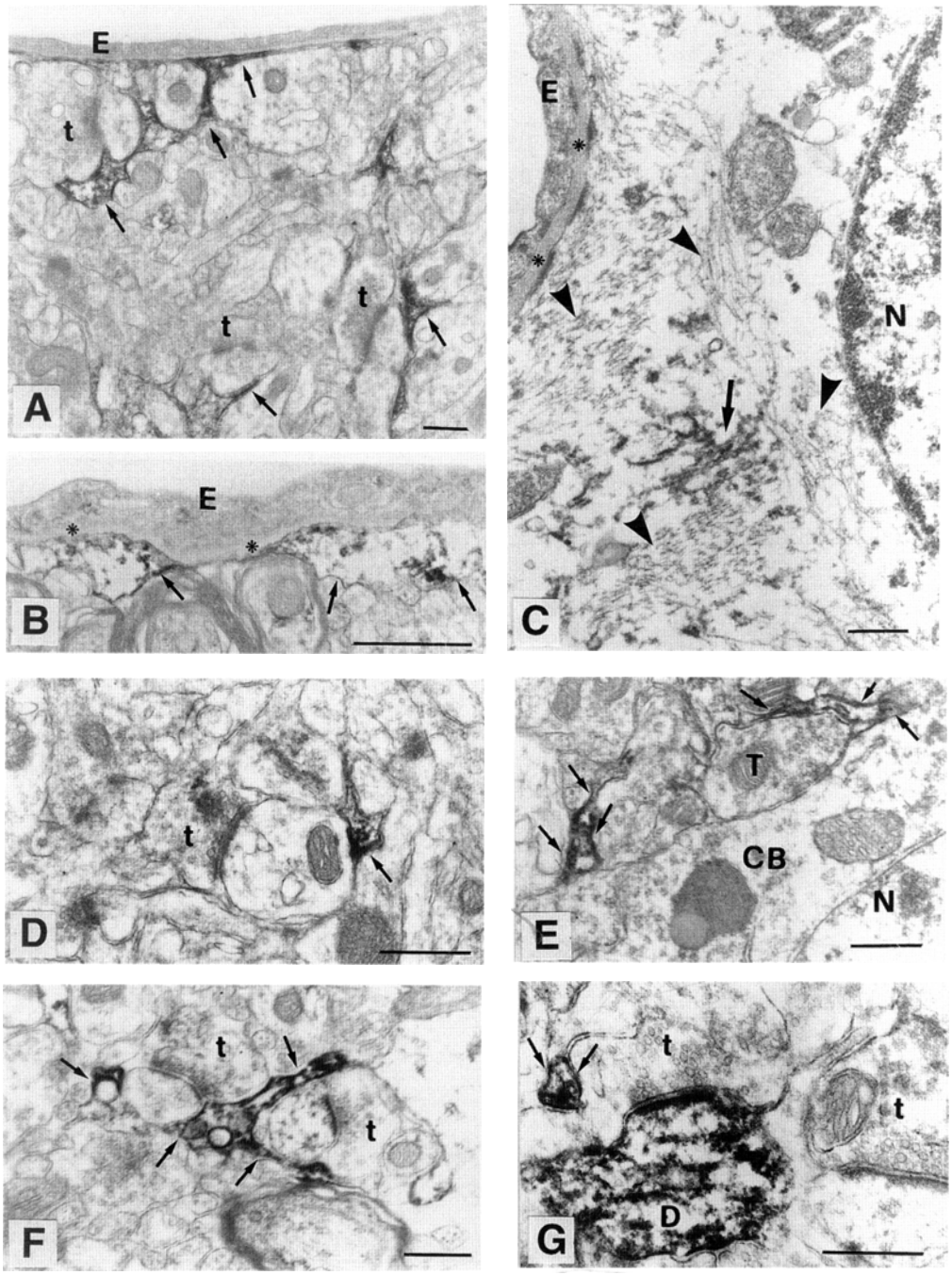

Fig. 9. NR1 (**A, C, D,** and **E**) and NR2A/B (**B, F,** and **G**) IR in cortical astrocytes. A: NR1+ astrocytic processes (arrows) beneath a capillary endothelium (E) and in the neuropil; t, axon terminals with asymmetric specialization. Lead citrate counterstaining. B: NR2A/B+ astrocytic process (arrows) beneath a capillary endothelium (E); asterisks indicate basal lamina. Unstained section. C: NR1+ astrocytic cell body beneath a capillary endothelium (E); arrow points to a clump of cytoplasmic reaction product; arrowheads, gliofilaments; N, nucleus; asterisks, basal lamina. Uranyl acetate and lead citrate counterstaining. D and E: NR1+ astrocytic processes (arrows) in the neuropil; t, axon terminal with symmetric specialization; CB cell body; N, nucleus. *(continued)*

Functional Implications

Notwithstanding the limitations inherent to each of the anatomical techniques employed to generate the data reported here (which have been discussed in the original publications), the availability of specific probes has made it possible to define with sufficient detail the basic features of the pattern of NMDA receptor localization in the mammalian cerebral cortex. Most importantly, the picture emerging from these studies allows inferences to be made on the physiological role of NMDA receptors in several aspects of cortical functions.

The outstanding features of NMDA receptor localization and their functional implications can be summarized as follows:

1. **NMDA receptors are present in many, but not in all neurons of the cerebral cortex.** The widespread distribution of NMDA receptors demonstrated in anatomical studies (Aoki et al., 1994; Conti et al., 1994a, 1996; Huntley et al., 1994; Conti and Minelli, 1996) is consistent with the predominant role of excitatory amino acid-mediated synaptic transmission in the cerebral cortex and with the tremendous impact of NMDA receptors activation on cortical functions (Collingridge and Watkins, 1994; Conti and Hicks, 1996). Following the original study of Moriyoshi et al. (1991), who reported that "(NMDAR1)...mRNA is expressed in almost all the neuronal cells throughout the brain regions" (p. 36), it has become customary to assume that virtually all cells express NMDA receptors. Although the semiquantitative analyses performed so far in the cerebral cortex on *in situ* hybridization and immunocytochemical material (Conti et al., 1994a, 1996; Conti and Minelli, 1996) are probably not devoid of sampling and methodological biases, the population of cortical neurons not expressing NMDA receptors is likely to be much larger than previously assumed.

   Both *in situ* hybridization and immunocytochemical studies indicate that neurons expressing NMDA receptors appears to be less numerous in layer IV than in layers II–III and V–VI (Conti and Minelli, 1996; *see also* 4 below). Given that afferent input reaches neocortex through Gluergic axonal endings mostly in layer IV (Kharazia et al., 1994), this observation is in line with previous suggestions that the bulk of thalamocortical transmission is not mediated by NMDA receptors (Tsumoto et al., 1986; Hagihara et al., 1988; Shirokawa et al., 1989; Armstrong-James et al., 1993; Weinberg and Kharazia, 1996), and suggests that the impact of NMDA receptor activation on cortical function is more important in later rather than in early stages of cortical processing.
2. **In cortical neurons, NMDA receptors are mostly formed by NR1 and NR2A and/or B subunits.** This is suggested by the results of several investigations showing that:
   a. NR1, NR2A, and NR2B are highly expressed, whereas NR2C and D are weakly expressed (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992, 1994; Nakanishi, 1992; Ishii et al., 1993; Aoki et al., 1994; Conti et al., 1994a; Huntley et al., 1994; Petralia et al., 1994a,b; Wenzel et al., 1995; Conti and Minelli, 1996);
   b. NR1 and NR2A and B exhibit similar distribution patterns, both at the light and at the electron microscopic level (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992, 1994; Ishii et al., 1993; Petralia et al., 1994a,b; Wenzel et al., 1995; Conti and Minelli, 1996);
   c. NR1 and NR2A/B IR are colocalized in most cortical neurons (Conti and Minelli, 1996); and
   d. "Triple subunit" heteromeric NMDA receptors (NR1 + NR2A + NR2B) are present in the cerebral cortex (Sheng et al., 1994).

   However, for the paucity of studies devoted to the analysis of NR2C and D expression in the cerebral cortex and for the reasons described earlier, it is presently impossible to rule out firmly a contribution, although a minor one, of NR2C and D.

Fig. 9 *(continued from opposite page)* Uranyl acetate and lead citrate counterstaining. F and G: NR2A/B-labeled astrocytic processes (arrows) in the neuropil; t, axon terminals with asymmetric specialization; D, labeled dendrite. Lead citrate counterstaining. Bars: 0.5 μm. From Conti et al. (1996).

Given that NR2C and D determine important biophysical properties (Monyer et al., 1994; Molinoff et al., 1994), it follows that the functional properties of cortical NMDA receptors cannot be inferred on the basis of present knowledge.

3. **The large majority of NMDA receptors are located postsynaptically on dendrites and dendritic spines.** The observation that NR1 and NR2A/B IRs are mostly present on dendrites and dendritic spines is in line with:
   a. The location of axon terminals forming asymmetric synapses (Peters, 1987) and the nature and location of Glu+ axon terminals (DeFelipe et al., 1988; Conti et al., 1989);
   b. Previous indications from radioligand binding (Monaghan et al., 1983, 1989; Greenamyre et al., 1985; Monaghan and Cotman, 1985; Maragos et al., 1988) and *in situ* hybridization (Moriyoshi et al., 1991; *see* Conti et al., 1994a for a discussion) studies; and
   c. The results of electrophysiological (e.g., Jones and Baughman, 1988) and combined electrophysiological Ca^{2+} imaging investigations (e.g., Bliss and Collingridge, 1993). Overall, this evidence indicates that the bulk of the effects of NMDA receptor activation is generated at distal dendrites and spines, and supports the view expressed by several authors that dendritic spines in cortical neurons are the site of biophysical events underlying complex integrative properties of cortical neurons (Crick, 1982; Sheperd et al., 1989; Jaslove, 1992; Gold and Bear, 1994; Yuste and Denk, 1995).
4. **NMDA receptors are preferentially expressed by pyramidal neurons.** Analysis of the morphology of NR1 and NR2A/B+ neurons showed that in rat neocortex, the large majority (about 70%) of all labeled neurons are pyramidal, and that this proportion is higher in layers II, III, V, and VI (Aoki et al., 1994; Huntley et al., 1994; Conti and Minelli, 1996). It is possible that these numbers reflect an indiscriminate staining of cortical neurons, and that the ratio of NR1 or NR2A/B+ pyramidal to nonpyramidal neurons simply expresses the ratio of all pyramidal to all nonpyramidal neurons. Although this possibility cannot be totally ruled out, given the uncertainty of the real number of pyramidal neurons (Feldman, 1984; DeFelipe and Farinas, 1992), the following considerations lend support to this conclusion:
   a. As reported above, the classification of neurons as pyramidal was based on strict criteria (Conti et al., 1987, 1992). We have previously provided evidence that many neurons that at light microscopic level were classified as nonpyramidal using these criteria had ultrastructural features of pyramidal neurons (Conti et al., 1989). This suggests that the actual proportion of NR1 and NR2A/B+ pyramidal neurons is much higher than that reported above;
   b. The percentage of NR1 and NR2A/B+ neurons is highest in those layers in which pyramidal neurons predominate;
   c. As discussed in the preceeding paragraph, NMDA receptors are preferentially located on dendritic spines, which is a typical, though not exclusive, attribute of pyramidal neurons (Cajal, 1911; Feldman, 1984; DeFelipe and Farinas, 1992); and
   d. Thomson and collaborators studied excitatory synaptic connections between pairs of cortical neurons recorded in cortical slices from adult rats, and characterized the receptor(s) mediating excitatory postsynaptic potentials (EPSPs; Thomson and Deuchars, 1994). They showed that connections between pyramidal neurons exhibit properties typical of NMDA-mediated processes, even though they are not exclusively mediated by NMDA receptors (Thomson and West, 1993; Thomson et al., 1993, 1996; Deuchars et al., 1994).

Although extreme caution is needed in comparing results obtained with different techniques on samples that differ by several orders of magnitude, those results are in line with the present conclusion by indicating that in all likelihood NMDA receptors display a preferential role in relation to pyramidal neurons.

Thomson and collaborators also showed that EPSPs evoked in (presumably inhibitory) interneurons on pyramidal neuron stimulation are mediated by "non-NMDA" receptors (Thomson et al., 1993). The data reported here show that nonpyramidal neurons too express NMDA receptors. Excitatory input to nonpyramidal neurons arises from pyramidal neurons (intrinsic, associa-

tion, and commissural), thalamocortical axon terminals, and spiny stellate cells (Peters, 1987). Since thalamocortical EPSPs do not appear to be mediated by NMDA receptors (Tsumoto et al., 1986; Hagihara et al., 1988; Shirokawa et al., 1989; Armstrong-James et al., 1993; Weinberg and Kharazia, 1996), NMDA receptors on nonpyramidal neurons must mediate either the connection between spiny and smooth nonpyramidal neurons or that from pyramidal to nonpyramidal neurons. Given that the targets of spiny stellate cells' axons are commonly dendritic spines and shafts of spiny dendrites, i.e., spiny stellate or pyramidal neurons (LeVay, 1973; Somogyi, 1978; Saint-Marie and Peters, 1985), then most NMDA receptors on nonpyramidal neurons must mediate input from pyramidal neurons. The difference between this conclusion and that of Thomson et al. (1993) presumably lies in the small sample studied electrophysiologically.

5. **Some NMDA receptors are presynaptic auto- and heteroreceptors.** Some NR1-NR2A/B+ axon terminals form asymmetric synapses (DeBiasi et al., 1996; *see also* Aoki et al., 1994; Huntley et al., 1994; Petralia et al., 1994 a,b; Johnson et al., 1996), and given that these axon terminals are either Glu- or aspartate (Asp)-positive (DeFelipe et al., 1988; Conti et al., 1989), it follows that NMDA receptors in axon terminals forming asymmetric synapses are autoreceptors that can facilitate Glu (or Asp) release. An unexpected result of our studies has been the identification of NR1 and NR2A/B in some GABAergic axon terminals (DeBiasi et al., 1996), which provided the first evidence that some NMDA receptors are heteroreceptors. These data suggest that NMDA receptors play a role in the regulation of GABAergic transmission. Overall, these data are consistent with previous demonstrations that presynaptic NMDA receptors contribute to NMDA-mediated phenomena in other regions of the nervous system (Chernevskaya et al., 1991; Bustos et al., 1992; Sherman et al., 1992; Liu et al., 1994; Parnas et al., 1994; Siegel et al., 1994; Van Bocstaele and Colago, 1996).
6. **NMDA receptors are expressed by astrocytes.** Whereas the notion that cortical astrocytes express Glu receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (KA) types has been firmly established (Pearce, 1993; Gallo and Russell, 1995; Blankenfeld et al., 1995; Kirchhoff and Kettenmann, 1996, for reviews), there has been considerable debate on whether astrocytes express NMDA receptors (*see* Conti et al., 1996; Van Bockstaele and Colago, 1996). The demonstration by electron microscopic immunocytochemistry that some cortical astrocytes do indeed express NR1 (Aoki et al., 1994; Conti et al., 1994a, 1996) and NR2A/B (Conti et al., 1996) subunits of the NMDA receptor indicates that at least part of the effects of NMDA receptor activation in the cerebral cortex may well be owing to astrocytic receptors. In particular, it has been suggested that they can monitor Glu release by neighboring axon terminals (DeFelipe et al., 1988; Conti et al., 1989) of thalamic (Kharazia and Weinberg, 1994) and corticocortical (Conti et al., 1988) origin, as well as from axon collaterals of cortical Gluergic neurons (Conti et al., 1987), and that therefore they can mediate part of the neuron-glia signaling mechanisms that regulate gene expression, responses to pathological elevations of Glu levels of astrocytes, and that they may participate in the mechanism(s) subserving activity-dependent cortical plasticity (Conti et al., 1996).

Conclusions

The data reviewed in the preceding sections indicate that in the last few years much has been learned about the cellular and subcellular localization of NMDA receptors in the cerebral cortex. From the present analysis, it appears that in some cases, anatomical studies have been confirmatory of previous findings; in other cases, however, neuroanatomical studies appear to have disclosed features that had not been described using other techniques, and await for electrophysiological and/or pharmacological confirmation.

However, this information is not sufficient to understand completely the effects of NMDA receptor activation, since they depend on many additional factors, including the number of

NMDA receptors in a given site, the identity of the NR1 splice variants, the NR2 subunits that contribute to the receptor, the contiguity with other receptors, and the interaction with other molecules. Recent experimental work has been devoted to investigating the expression of NR1 splice variants (Laurie and Seeburg, 1994; Laurie et al., 1995; Johnson et al., 1996) and the colocalization of NMDA receptor subunits with AMPA receptor subunits at the cellular (Huntley et al., 1994) and synaptic levels (Kharazia et al., 1996), and with nitric oxide synthase (NOS; Aoki et al., 1996). These studies have allowed important insights to be gained into essential issues, e.g., the differential expression of NR1–1/NR1–4 splice variants (Laurie et al., 1995), the demonstration that axospinous synapses expressing postsynaptic NMDA receptors can be differentiated according to whether they also express AMPA receptor subunits (Kharazia et al., 1996), and the complex relationships between NOS+ and NMDA+ neural elements (Aoki et al., 1996). Although these studies are at present too scanty to allow any conclusions to be drawn, they are setting the scene for correlating the localization of NMDA receptors at cortical sites with their neurobiological effects and for the elucidation of their role in several neuropsychiatric disorders (e.g., Akbarian et al., 1996).

Acknowledgments

We wish to thank all the colleagues who participated in the studies cited in the references for their contributions; C. Aoki, G. Huntley, and R. Weinberg for sharing unpublished information, and R. J. Wenthold, S. Nakanishi, and V. N. Kharazia for kindly providing antipeptide antibodies, full-length NR2A and NR2B rat cDNAs and the photomicrographs of Fig. 5, in this order. Personal work described here has been supported by funds from Ministero dell'Università e della Ricerca Scientifica e Tecnologica, NATO, and Consiglio Nazionale delle Ricerche.

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