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Regional and Developmental Heterogeneity in Splicing of the Rat Brain NMDAR1 mRNA

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Developmental and regional alternative splicing of the NMDAR1 subunit gene transcript was examined by *in situ* hybridization in the developing and adult rat brain. NMDAR1 mRNA, barely detectable at embryonic day 14, increased gradually during development until the third postnatal week, after which it declined slightly to adult levels, when it was detected in every examined neuronal type. Each splice form of the primary NMDAR1 gene transcript was found to follow a parallel profile of abundance in the brain, but marked regional differences were observed in splicing at both 5′ and 3′ sequences. The individual regional distributions of splice forms appeared to be established around birth, with little change thereafter, except in the overall abundance. The NMDAR1-a and NMDAR1-2 splice forms occurred extensively and approximately homogeneously throughout brain gray matter. The NMDAR1-b variant was found primarily in the sensorimotor cortex, neonatal lateral caudate, thalamus, hippocampal CA3 field, and cerebellar granule cells, but was absent from adult caudate. The NMDAR1-1 and -4 splice forms were detected in almost complementary patterns; the former was concentrated in more rostral structures such as cortex, caudate, and hippocampus, while the latter was principally in more caudal regions such as thalamus, colliculi, and cerebellum. These two splice forms accounted for a greater proportion of the adult NMDAR1 mRNA than that of the neonate. The NMDAR1-3 mRNA variant was scarce, being detected only at very low levels in postnatal cortex and hippocampus. The different splice forms may generate regional differences in NMDA receptor properties during development and in the adult CNS.

[Key words: NMDA receptor, NMDAR1 mRNA,* in situ *hybridization, splice variants, rat brain, development]

The NMDA-selective type of cerebral glutamate ionotropic receptor is believed to have important roles in neuron differentiation and synapse consolidation in the developing brain (McDonald and Johnston, 1990; Yuste and Katz, 1991), and long-term potentiation (LTP), synaptic plasticity, and neurotoxicity in the adult brain (Collingridge and Singer, 1990; Meldrum and Garthwaite, 1990). Two families of subunits of this receptor have recently been cloned from rat brain and termed NMDAR1 and NMDAR2 (Moriyoshi et al., 1991; Monyer et al., 1992). The equivalent murine families are called ζ and ϵ, respectively (Kutsuwada et al., 1992; Meguro et al., 1992; Yamazaki et al., 1992). Several related subunits of the second family have been found (NMDAR2A to NMDAR2D) that, when coexpressed with NMDAR1, form glutamate-gated ion channels, with distinct electrophysiological properties and ligand affinities (Kutsuwada et al., 1992; Monyer et al., 1992; Nakanishi, 1992). Functional diversity in the related family of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors is created by both subunit variety and alternative splicing (Keinänen et al., 1990; Sommer et al., 1990). After the initial cloning of the NMDAR1 sequence followed several reports of forms of this subunit apparently arising by alternative splicing of a single gene transcript (Anantheram et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992; Yamazaki et al., 1992; Hollmann et al., 1993). These reports described the insertion of a splice cassette into the proposed amino-terminal region of the original NMDAR1 sequence (termed as the 5′ insertion) and the deletion of two independent consecutive splice cassettes (3′ deletions 1 and 2) from the carboxy terminus, the second of which also replaces the original carboxy terminus with a new sequence (see Fig. 1*a*). These alterations can occur individually or together, since each of the eight possible combinations have been isolated in differing proportions from rat brain (Sugihara et al., 1992; Hollmann et al., 1993). In this study we have followed the terminology of Hollmann et al. (1993), who named the splice variants NMDAR1-1a, NMDAR1-1b, NMDAR1-2a, and so on, where the second number indicates the splice variant at the 3′ end (1 = no deletion; 2, 3, and 4 = deletions 1, 2, 1 + 2, respectively), and "a" and "b" indicate the absence or presence, respectively, of the 5′ insertion.

The functional consequences of NMDAR1 mRNA splicing are as yet incompletely characterized, but results from studies on homomeric channels indicate differences in agonist and antagonist potency, polyamine sensitivity, Zn^{2+} responses, ethanol sensitivity, and phosphorylation and potentiation by protein kinase C (PKC) activators (Anantheram et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992; Yamazaki et al., 1992; Hollmann et al., 1993; Tingley et al., 1993). The members of the NMDAR2 subunit family are differentially expressed in developing and adult rodent brain, in contrast to the total NMDAR1 mRNA, which is abundantly and ubiquitously expressed (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Monyer et al., 1992; M. Watanabe et al., 1992; H. Monyer, N. Burnashev, D. J. Laurie, B. Sakmann, and P. H. Seeburg, unpublished observations). RNase protection and *in situ* hybrid-

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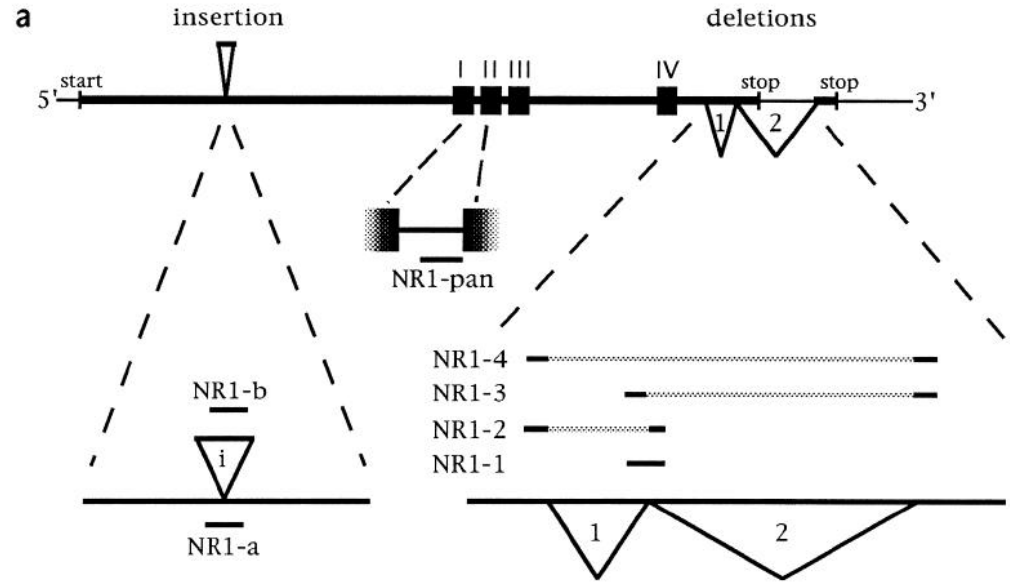

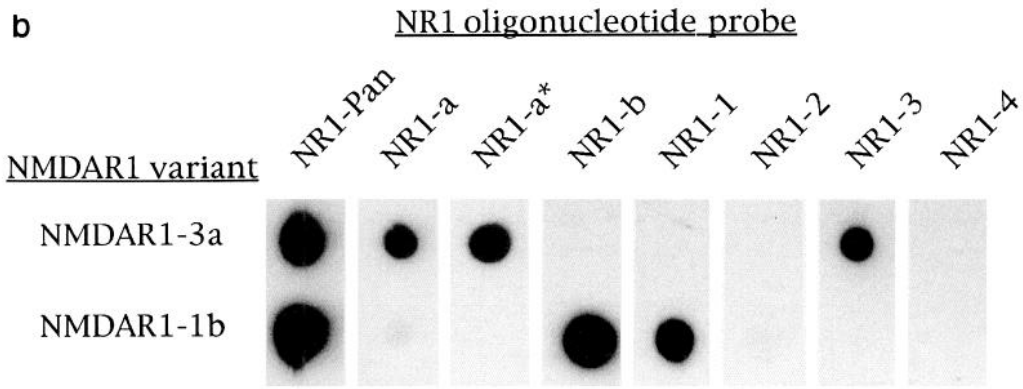

Figure 1. Oligonucleotide probes. *a*, Schematic representation of the NMDAR1 mRNA, showing transmembrane domains I–IV, the 5′ insertion, 3′ deletions 1 and 2, the start codon, and the two alternative stop codons (Sugihara et al., 1992; Hollmann et al., 1993). The complementary positions of the splice-specific antisense oligonucleotide probes are shown as *short solid lines*; the *stippled lines* represent intervening sequences not included in the probes. *b*, Autoradiograph showing the hybridization of ^{32}P 5′-labeled probes to two specific rat NMDAR1 plasmid DNAs (50 fmol each dot) immobilized on nitrocellulose. See Materials and Methods for description of probes.

ization analyses have indicated that splicing of the N-terminal cassette is regionally regulated in adult rat brain (Nakanishi et al., 1992; Standaert et al., 1993), suggesting regional heterogeneity in function may exist. Such heterogeneity may also be temporally regulated, giving rise to different splice variant populations and differing responses at various stages of development. By *in situ* hybridization in developing and adult rat brain with probes specific for the various splice variant mRNAs, we have found that splicing of all three cassettes is both regionally and developmentally regulated.

Materials and Methods

The experimental procedures employed for *in situ* hybridization were as described by Wisden et al. (1991), Wisden and Morris (1994), and Laurie and Schrotz (1994). Nonperfused rat brains or whole rat embryos were removed, frozen on dry ice, and sectioned (14 μm) on a microtome. Hybridization was performed on sagittal sections of whole embryos of gestational ages 14, 17, and 19 d (E14, E17, and E19), and on horizontal or sagittal sections of brain from rats of postnatal ages 0, 7, and 12 d (P0, P7, P12) and adult. Gender was only determined for the adult male rats. Three animals at each developmental stage were examined.

Antisense oligonucleotide probes were 3′ end labeled to a specific activity of approximately 15 μCi/pmol using terminal deoxynucleotidyl transferase (Boehringer) and a 30:1 molar ratio of α-^{35}S-dATP (1200Ci/mmol; New England Nuclear) to probe. The probe recognizing all NMDAR1 mRNAs (NR1-pan) was the 45-mer described by Monyer et al. (1992), which hybridizes to the NMDAR1 mRNA sequence encoding the region between transmembrane domains I and II. The other oligonucleotide probes (40-mers) were designed against specific sequences of the original NMDAR1 clone (Moriyoshi et al., 1991) at the splice sites (Fig. 1*a*). They were named according to the splice variants to which they would hybridize. For example, NR1-a is complementary to the 5′ splice region in NMDAR1-1a, -2a, -3a and -4a mRNAs, while NR1-3 recognizes both NMDAR1-3a and -3b (see Fig. 1*a*). The probe sequences were as follows: NR1-a, AACTGCAGCACCTTCTCTGC-CTTGGACTCCCGTTCCTCCA (bases 551–590; Moriyoshi et al., 1991); NR1-b, GCGCTTGTTGTCATAGGACAGTTGGTCGAGG-TTTTCATAG (bases 15–54 of insert; Sugihara et al., 1992); NR1-1, TCCACCCCCGGTGCT-CGTGTCTTTGGAGGACCTACGTCTC (bases 2676–2715); NR1-2, TCCACCCCCGGTGCT-CTGCAGGTTC-TTCCTCCACACGTTC (bases 2565–2715); NR1-3, GATATCAGTG-

GGATGGTACTG–CGTGTCTTTGGAGGACCTA (bases 2682–3077); and NR1-4, GATATCAGTGGGATGGTACTG–CTGCAGGTTCTTCCTCCAC (bases 2574–3077). Dashes indicate where probes span a splice site (see Fig. 1*a*). The maximum consecutive length of oligonucleotide capable of hybridizing to the wrong splice form was 19–25 bases, which under the stringent conditions of hybridization [50% formamide, 4× saline-sodium citrate (SSC), 10% dextran sulfate, 42°C, overnight] and washing (1× SSC, 60°C, 20 min) is very unlikely to result in cross-hybridization (Wisden et al., 1991; Wisden and Morris, 1994) (see below). After hybridization and washing, sections were apposed to x-ray film (Kodak X-Omat SB-5) for 10 d, and then dipped in photographic emulsion (Ilford K5) and exposed for 5 weeks. The anatomy of autoradiographs and thionin-stained sections was assessed from the atlases of Paxinos and Watson (1986), Paxinos et al. (1991), and Swanson (1992). Microscopic examination was performed for every structure described. Photomicrographs were obtained using a Zeiss Axioplan microscope under light- and dark-field optics.

Specificity of the hybridization signals was determined in several ways.

(1) Parallel competition experiments, in which excess (200-fold) unlabeled probe was added to the hybridization buffer, resulted in virtually blank autoradiographs (data not shown) except for some nonspecific signals from peripheral tissues such as embryonic liver and thymus (Figs. 2–4) (see Laurie and Schrotz, 1994).

(2) When labeled NR1-2 or NR1-4 probes were each incubated with a large excess (200-fold) of the other, unlabeled oligonucleotide only a slight reduction of signal was observed (data not shown). When compared to the result of control (item 1 above), this indicates only very limited cross-hybridization.

(3) Each probe was 5′ labeled using γ-^{32}P-ATP (6000 Ci/mmol; New England Nuclear) and polynucleotide kinase to a specific activity of 2.5 μCi/pmol. Aliquots (0.05–50 fmol) of two plasmid DNAs carrying the NMDAR1-1b and NMDAR1-3a splice versions were dotted and immobilized on nitrocellulose membranes, and incubated overnight with each probe. Hybridization and washing conditions were the same as those described above for tissue sections. The washed membranes were exposed to film for 1 hr. Each probe was found to hybridize strongly to the appropriate plasmid (Fig. 1*b*) with only slight cross-hybridization detected for the NR1-a and NR1-2 oligonucleotides at the NMDAR1-1b dot. In order to reproduce more closely the conditions of the *in situ* hybridization procedure, 5 pmol of the NR1-a probe was 3′ tailed with nonradioactive dATP before being 5′ labeled with ^{32}P-ATP and hybridized to immobilized plasmids as described above. This probe was termed NR1-a*. Cross-hybridization was considerably further reduced by the presence of the 3′ polyadenosine tail (Fig. 1*b*), and was only detectable after an overnight exposure (not shown).

Results

The changes in signal strength for each splice-specific probe indicate that splicing of the NMDAR1 mRNA is regulated both regionally and developmentally. All signals reached a peak in the third postnatal week and subsequently declined to adult levels, but each probe exhibited an idiosyncratic hybridization pattern, indicating regional and age-dependent populations of the NMDAR1 subunit splice variants.

E14

E14 was chosen as a starting point as this stage is prior to generation, migration, and differentiation of most telencephalic and mesencephalic neurons (Jones 1985; Jacobson, 1991). Hybridization of the NR1-pan probe indicated that the mRNA was expressed weakly throughout the E14 CNS, slightly favoring the colliculi and ventral spinal cord (Fig. 2). Similar, weaker signal patterns were obtained with the other probes except NR1-3, which gave no detectable signal at any examined embryonic stage. Hybridization signals in the embryonic liver and thymus (Figs. 2–4) were also observed in competition controls, and are therefore nonspecific.

E17 and E19

At these stages the total NMDAR1 mRNA was detected at a moderate level throughout the brain, with stronger hybridization in the spinal cord, thalamus, and layer I of the developing cortex (Figs. 3, 4). Signals were also observed in dorsal root ganglia, the nasal epithelium, and the trigeminal ganglion. The NR1-a and NR1-2 probes produced images similar to that of NR1-pan (Figs. 3, 4), indicating that the NMDAR1-2a form predominates at this stage. The strongest signals for NR1-b and NR1-1 in the brain were found in the thalamus, colliculi, and cortical layer I, whereas the NR1-4 probe hybridized mainly to the tegmentum and pons. All probes except NR1-3 gave stronger signals in the spinal cord, where a greater variety of splice forms is therefore inferred to exist. Hybridization was greater in ventral spinal cord regions except for the NR1-1 probe. No signal for any probe was detected in the cortical plate.

P0

The NR1-pan probe showed that at birth total NMDAR1 mRNA is abundant throughout the brain gray matter, especially in hippocampus and outer layers of the cerebral cortex (Fig. 5). One exception to the ubiquitous strong expression is the globus pallidus, which was not as highly decorated with silver grains. The NMDAR1-a splice form is similarly distributed at a moderate level, whereas the NMDAR1-b mRNA is concentrated in the thalamus, sensorimotor cortex, and brainstem. Hybridization patterns of the deletion variant probes began to be more easily discernable than previously. The NR1-2 probe gave a strong, approximately homogeneous signal, similar to that obtained with the NR1-a probe. In contrast, the NMDAR1-1 and NMDAR1-4 splice variants were more weakly detected, NMDAR1-1 being concentrated in the cortex, septum, caudate, hippocampus, and anterior thalamus, and NMDAR1-4 in posterior thalamus, colliculi, and brainstem. The NMDAR1-3 form was first observed at P0 at a barely detectable level, primarily in cortex and hippocampus. All probes hybridized to the Purkinje cell layer except NR1-3. The NR1-pan, NR1-a, and NR1-1 probes hybridized weakly to the external layer of premigratory granule cells.

P7

The different patterns of forebrain NMDAR1 mRNA splicing observed at P0 became more consolidated by P7, as the total amount of NMDAR1 mRNA increased (Fig. 6). Hybridization with the NR1-a and NR1-2 probes indicated that the NMDAR1-2a mRNA was predominant and widespread, both probes giving intense signals in hippocampus and olfactory bulb, and strong signals elsewhere, except the globus pallidus. At this age, migratory cortical neurons have reached their final destination (Jacobson, 1991). No marked lamination of the cortical signal was observed, but layers II, III, and V were slightly more intense than the others. NMDAR1-b mRNA was much more restricted, with hybridization highlighting some thalamic nuclei, olfactory bulb, lateral striatum, CA3 of the hippocampus, inferior colliculi, brainstem, and layers III and V of the sensorimotor and parietal cerebral cortices. Unlike the NR1-2 signal, which was strong throughout the forebrain, that of NR1-1 was principally in more peripheral structures such as cortex, striatum, hippocampus, dorsal septum, and olfactory bulb, while that of NR1-4 was concentrated in deeper structures like ventral septum, thalamus, colliculi, brainstem, and hippocampal CA3 field, in ad-

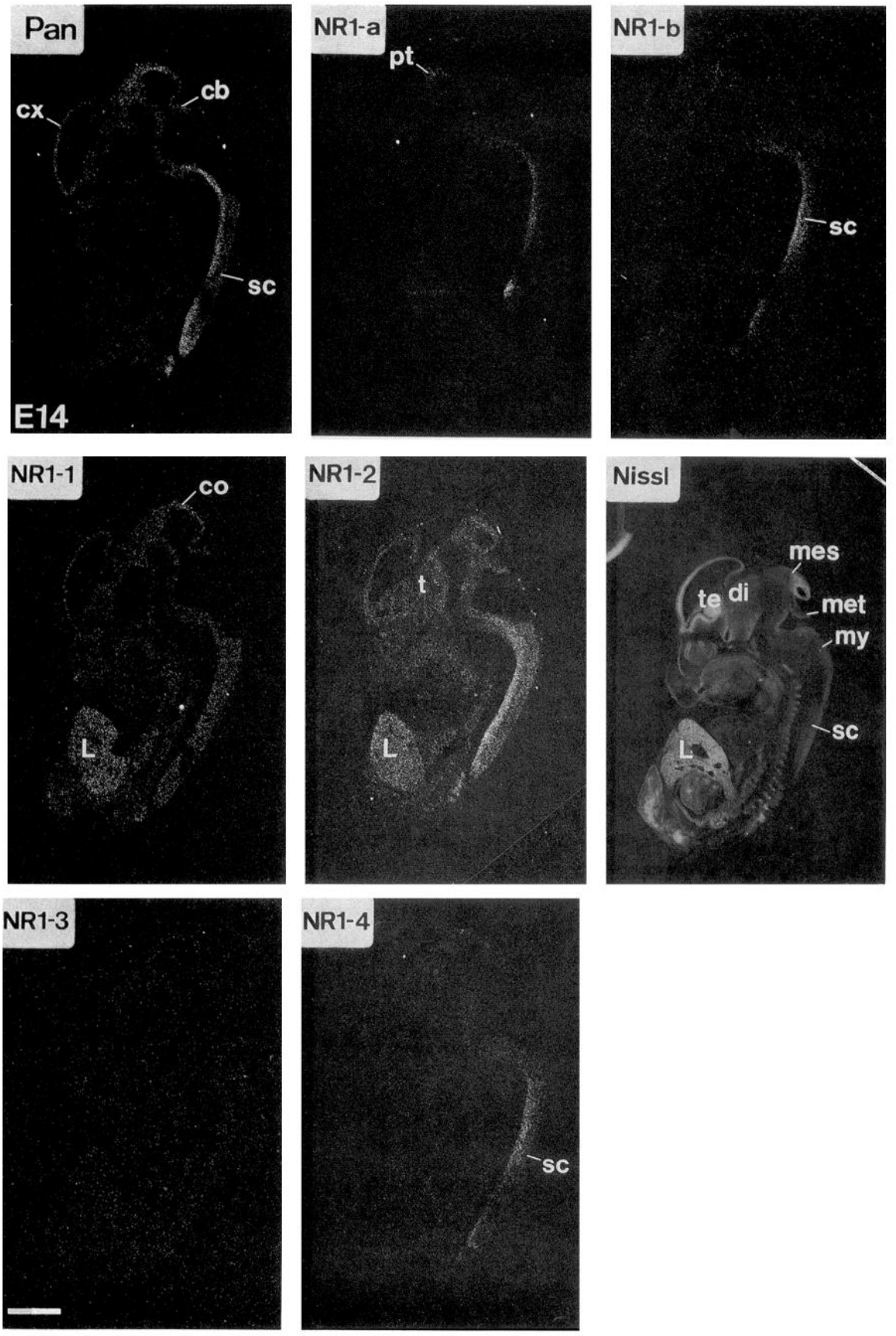

Figure 2. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to sagittal sections of E14 rat embryos. *Pan*, NR1-pan; *Nissl*, Nissl stain. For abbreviations, see Appendix. Scale bar, 2 mm.

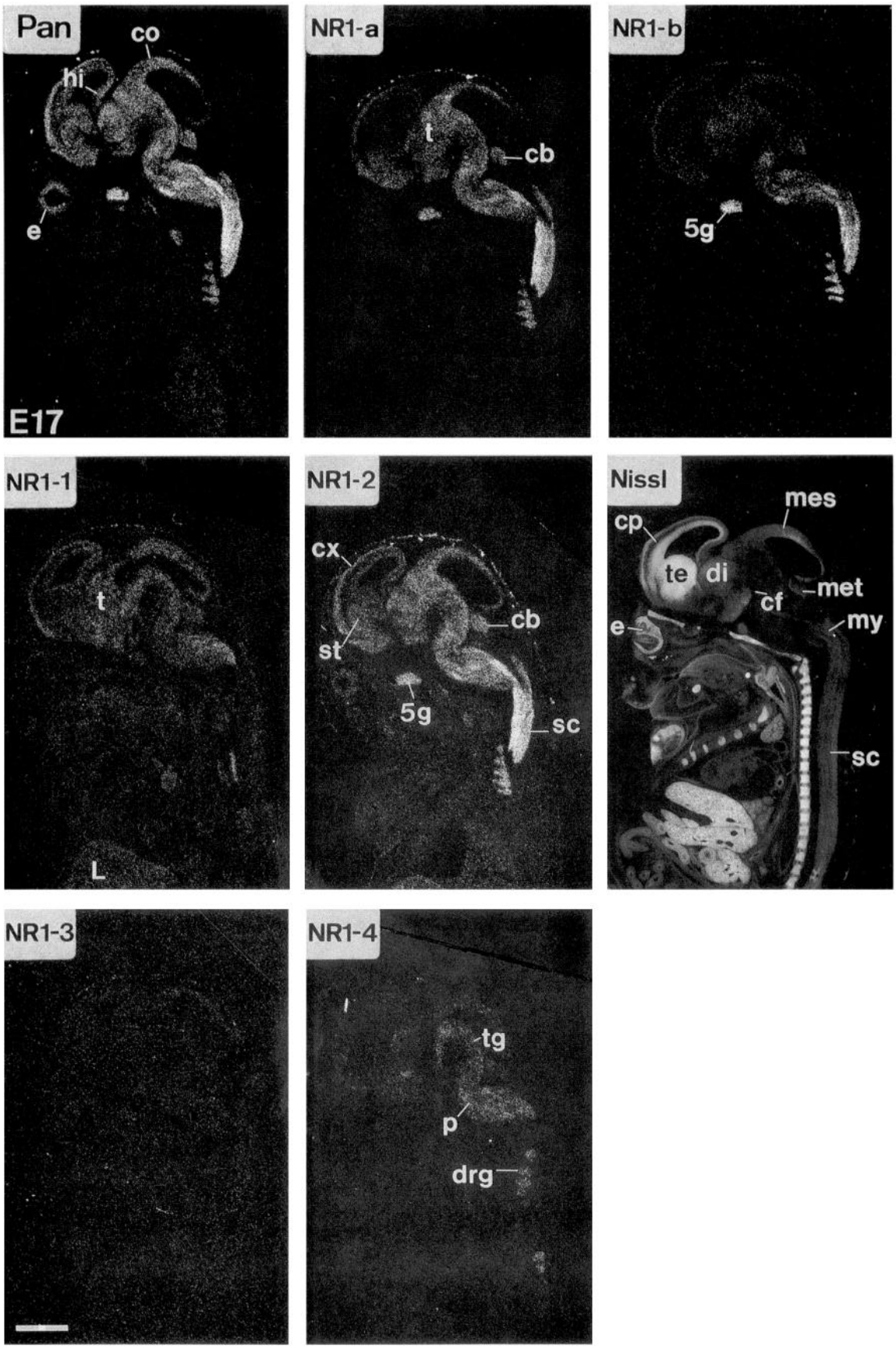

Figure 3. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to sagittal sections of E17 rat embryos. *Pan*, NR1-pan; *Nissl*, Nissl stain. For abbreviations, see Appendix. Scale bar, 2 mm.

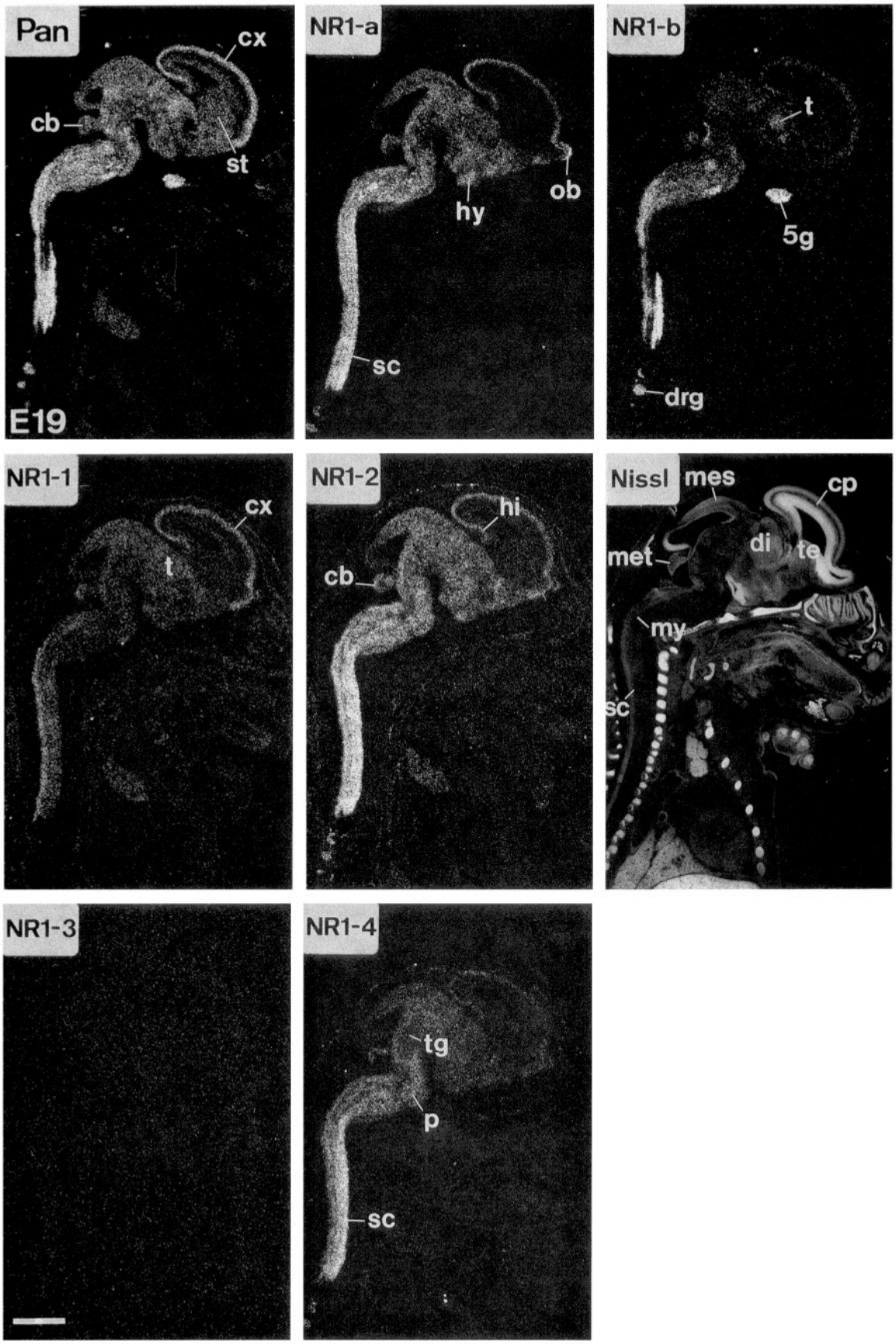

Figure 4. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to sagittal sections of E19 rat embryos. *Pan*, NR1-pan; *Nissl*, Nissl stain. For abbreviations, see Appendix. Scale bar, 2 mm.

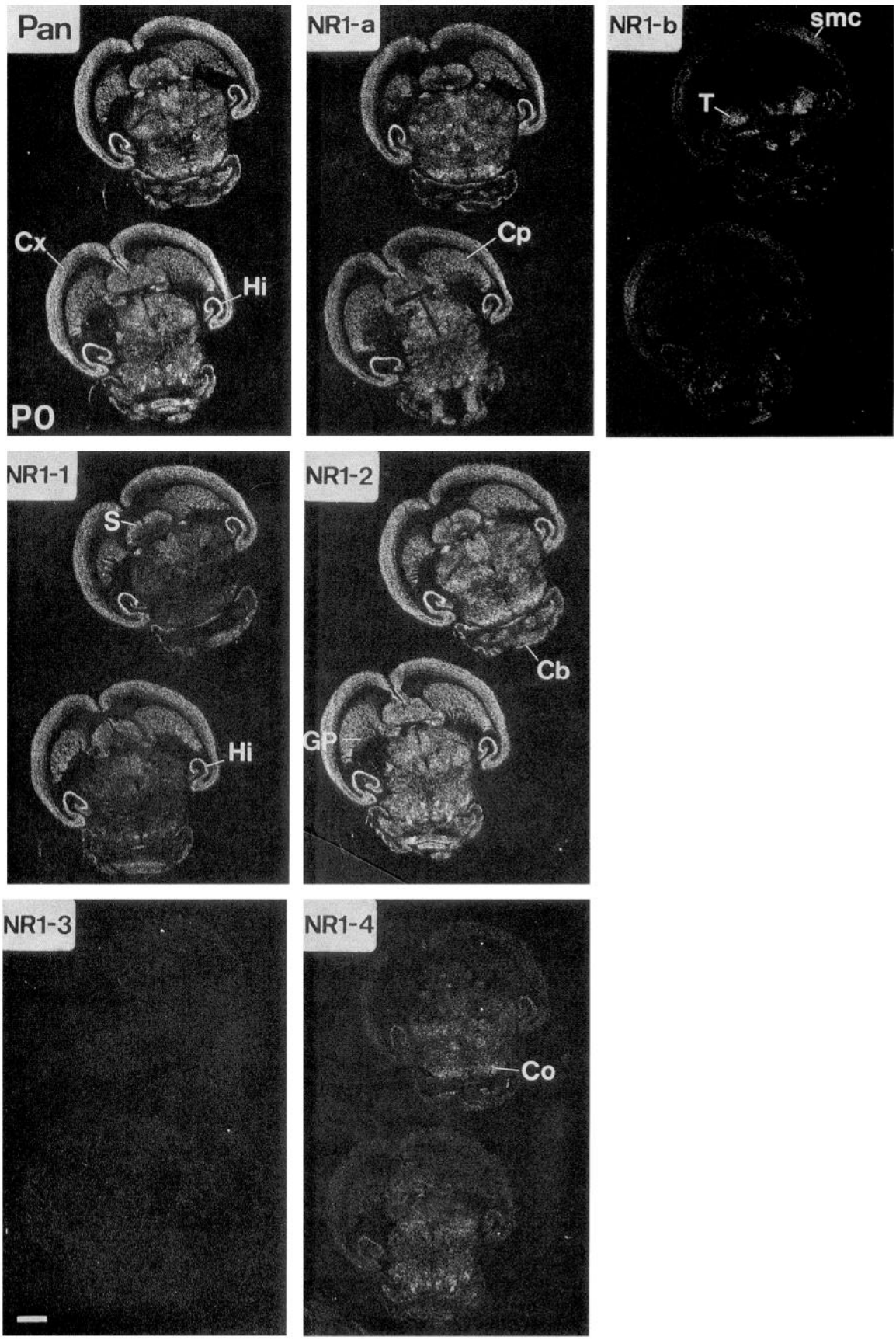

Figure 5. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to horizontal sections of P0 rat brain. *Pan*, NR1-pan. For abbreviations, see Appendix. Scale bar, 1.8 mm.

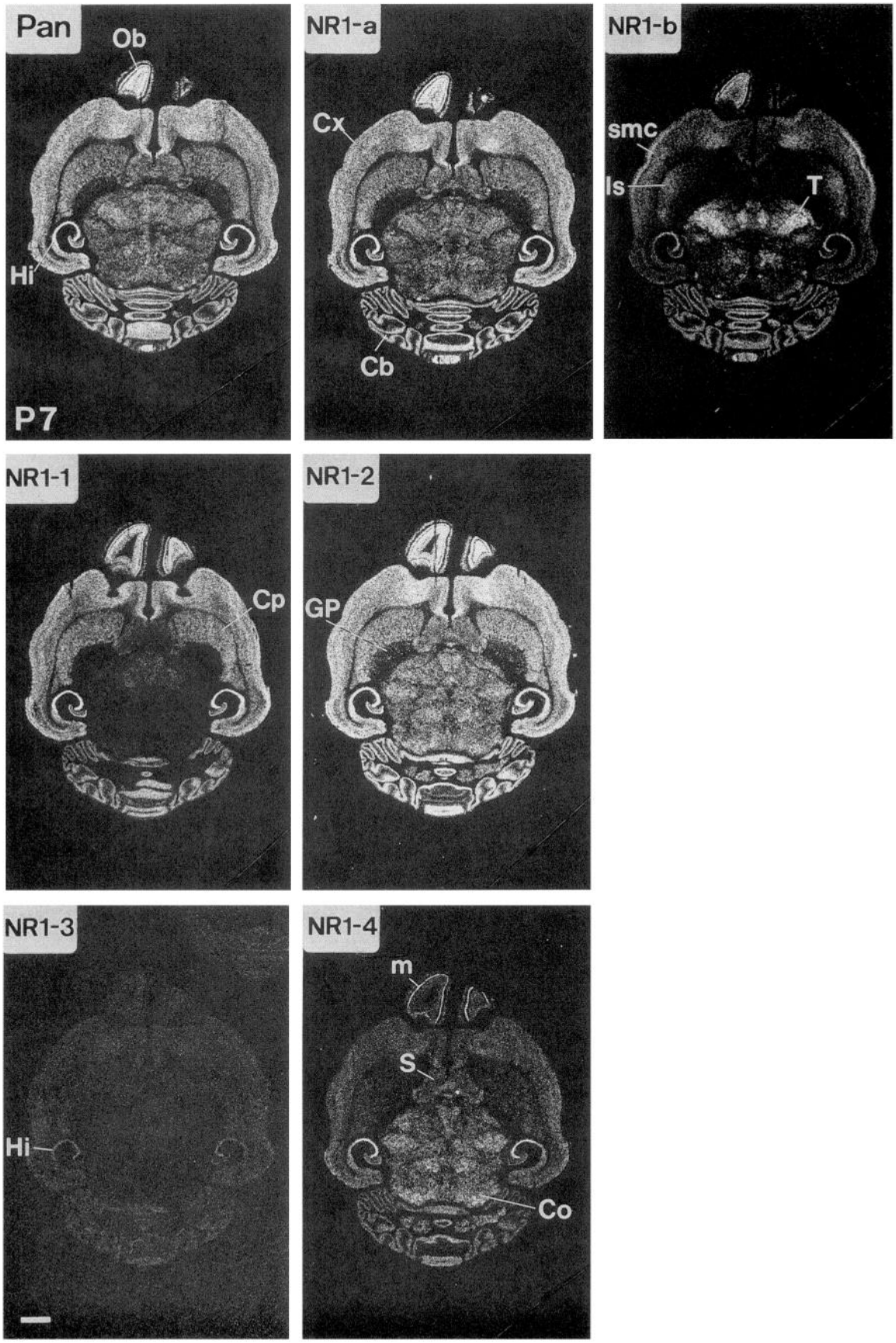

Figure 6. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to horizontal sections of P7 rat brain. *Pan*, NR1-pan. For abbreviations, see Appendix. Scale bar, 1.8 mm.

dition to mitral cells of the olfactory bulb. Only a minor proportion of the forebrain NMDAR1 mRNA at P7 was present as the NMDAR1-3 splice form, the NR1-3 probe giving a very weak signal in cortex, thalamus, and hippocampus. In the cerebellum, the NR1-pan, -a, and -2 probes each strongly labeled both postmigratory granule cells and Purkinje cells, while NR1-b labeled both moderately, and NR1-3 weakly labeled only granule cells. Although both NR1-1 and NR1-4 probes hybridized to both cell types, the first probe principally labeled granule cells and the second Purkinje cells. Weak hybridization to migrating and immediately premigratory granule cells was observed for all probes except NR1-3 and NR1-4.

P12

All probes gave their strongest hybridization signals at P12. This study did not address the stage of peak expression for each splice form, but we have found from *in situ* hybridization data (D. J. Laurie and P. H. Seeburg, unpublished observations) that the peak of NMDAR1 mRNA expression, and of splicing within the N-terminal coding sequences, occurs between P14 and P20. For each splice-specific probe, patterns of hybridization were regionally very similar to that described above for P7, despite the increase in abundance. However, the relatively larger increases in NMDAR1-b, -1, -3, and -4 mRNA abundance indicate that these splice forms begin to account for a greater proportion of NMDAR1 mRNA in forebrain and cerebellum than previously (Fig. 7).

Adult

After the third postnatal week, the strength of all hybridization signals declined, although the different regional patterns of NMDAR1 mRNA splicing were fundamentally preserved (Figs. 8, 9). As has been described before (Moriyoshi et al., 1991; Monyer et al., 1992), the NMDAR1 mRNA was abundant in every adult neuronal type examined. Most of this mRNA was of the NMDAR1-a type, except in certain areas such as sensorimotor cortex, thalamus, hippocampal CA3 field, colliculi, olfactory bulb, pontine nuclei, and brainstem, where a considerable proportion of NMDAR1 mRNA contained the 5′ insertion. Differential splicing of this site was also observed in the cerebellum where the NMDAR1-a form predominated in Purkinje cells, and the NMDAR1-b form in stellate-basket cells and granule cells (Figs. 8–10), compared to the overall predominance of the NMDAR1-a type at earlier (P7) stages (Fig. 6). All Purkinje cells were lightly labeled by the NR1-b probe (Fig. 10). In contrast to the neonatal brain, where the NMDAR1-2 variant predominated considerably, an estimated half of adult brain NMDAR1 mRNA was of this form and was homogeneously expressed. The remainder appeared to comprise the NMDAR1-1 and -4 forms in almost complementary distributions, with the NMDAR1-1 form concentrated in rostral structures (e.g., olfactory bulb, cortex, caudate, hippocampus) and the NMDAR1-4 variant primarily in caudal areas (hippocampus, thalamus, colliculi, cerebellum, brainstem) (Figs. 8, 9). In further contrast to earlier (e.g., P7) stages, the NMDAR1-1 variant was only weakly detected in adult cerebellar granule cells, whereas moderate amounts of the NMDAR1-4 form were found in both Purkinje and granule cell types. The NR1-3 variant was, again, barely detectable in adult cortex and hippocampus. Hybridization signals from each probe except NR1-3 were unexpectedly detected over some cells in cortical layer I, which were presumably glia (Jacobson, 1991), but not over glial cells in brain white matter tracts.

Discussion

From this *in situ* hybridization study on developing rat brain we have observed that the primary NMDAR1 gene transcript is spliced at both 5′ and 3′ sequences in a regionally differential manner, and that these regional patterns of splicing do not greatly change between birth and adulthood, despite considerable changes in overall abundance. The caudal-to-rostral gradient in the appearance of embryonic NMDAR1 mRNA, the early postnatal peak and the ubiquitous, high neuronal adult expression levels concur with other studies on total NMDAR1 mRNA in developing and adult rodent brain (Moriyoshi et al., 1992; M. Watanabe et al., 1992). Similarly, the adult brain distribution of the NMDAR1-b mRNA form (Figs. 8–10) agrees very well with that described by recent *in situ* hybridization and RNase protection studies (Nakanishi et al., 1992; Standaert et al., 1993). The drop in the NR1-pan signal observed between P12 and adult is probably a consequence of both normal neuronal death (Oppenheim, 1991) and a reduction in expression by each neuron, since the grain clusters over individual neurons were much denser at P12 than at maturity (not shown).

The different patterns of hybridization to brain sections (Figs. 2–10) and to known NMDAR1 variants on nitrocellulose (Fig. 1*b*) indicate that each 40-mer probe recognized a specific splice variant despite cross-identity of up to 25 consecutive nucleotides (Fig. 1*a*). It would be expected that the total signals obtained by combining the NR1-a and -b images, or the NR1-1, -2, -3, and -4 images should be equivalent to that obtained with the NR1-pan probe. Allowing for factors such as the lower specific activity of the NR1-pan probe (approximately two-thirds of the others to avoid overprinting) and its slightly greater length, the NR1-pan signal does appear to be approximately the sum of each subgroup. The ratios of the NMDAR1-a and -b mRNA splice forms in adult rat forebrain and cerebellum, previously suggested to be about 5:1 and 1:5, respectively (Anantharam et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992), concur with visual estimates from the present *in situ* hybridization figures (Figs. 8, 9). However, there is less consensus over the relative amounts of the 3′ splice forms NMDAR1-1, -2, -3, and -4 in forebrain; ratios of 1.5:1:x:x and 9:1:0.8:0.8 have been proposed [Anantharam et al., 1992 (x = not reported); Sugihara et al., 1992]. Visual assessment of the present results implies relative amounts with the order NMDAR1-2 > -1 > -4 ≫ -3 (Figs. 8, 9). The different ratios may be due to the different methods employed (semiquantitative PCR, library screening).

Regional populations

Examination of the hybridization patterns reveals similarity between the images obtained using the NR1-b and -4 probes (Figs. 8, 9). The NR1-a and -1 probes also yielded largely overlapping hybridization patterns (Figs. 8, 9). These results, together with the widespread signals obtained with the NR1-2 probe, indicate that the NMDAR1-1a and -2a forms may frequently occur in cortex, caudate, and hippocampus, and the NMDAR1-2b and -4b forms in thalamus, colliculi, cerebellum, and brainstem. However, there exists considerable overlap between all splice variant images, so it will require other approaches (e.g., regional library screening, splice variant-specific antibodies) to ascertain regional populations. Use of such antibodies would also be useful in determining the cellular location of the splice forms. The different patterns of NMDAR1 mRNA splice forms are fundamentally constant throughout postnatal development, indications of future heterogeneity even being apparent as early as

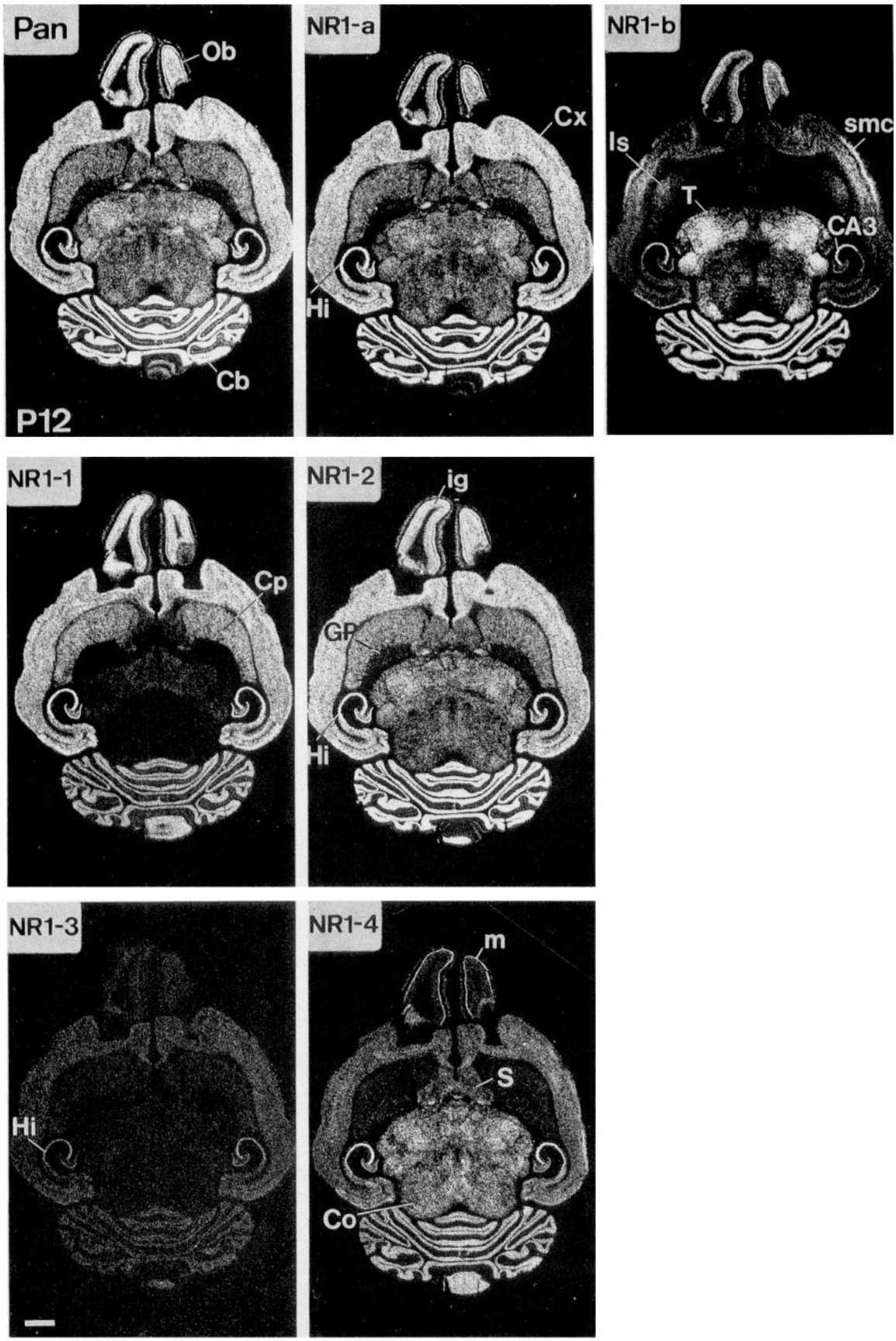

Figure 7. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to horizontal sections of P12 rat brain. *Pan*, NR1-pan. For abbreviations, see Appendix. Scale bar, 1.8 mm.

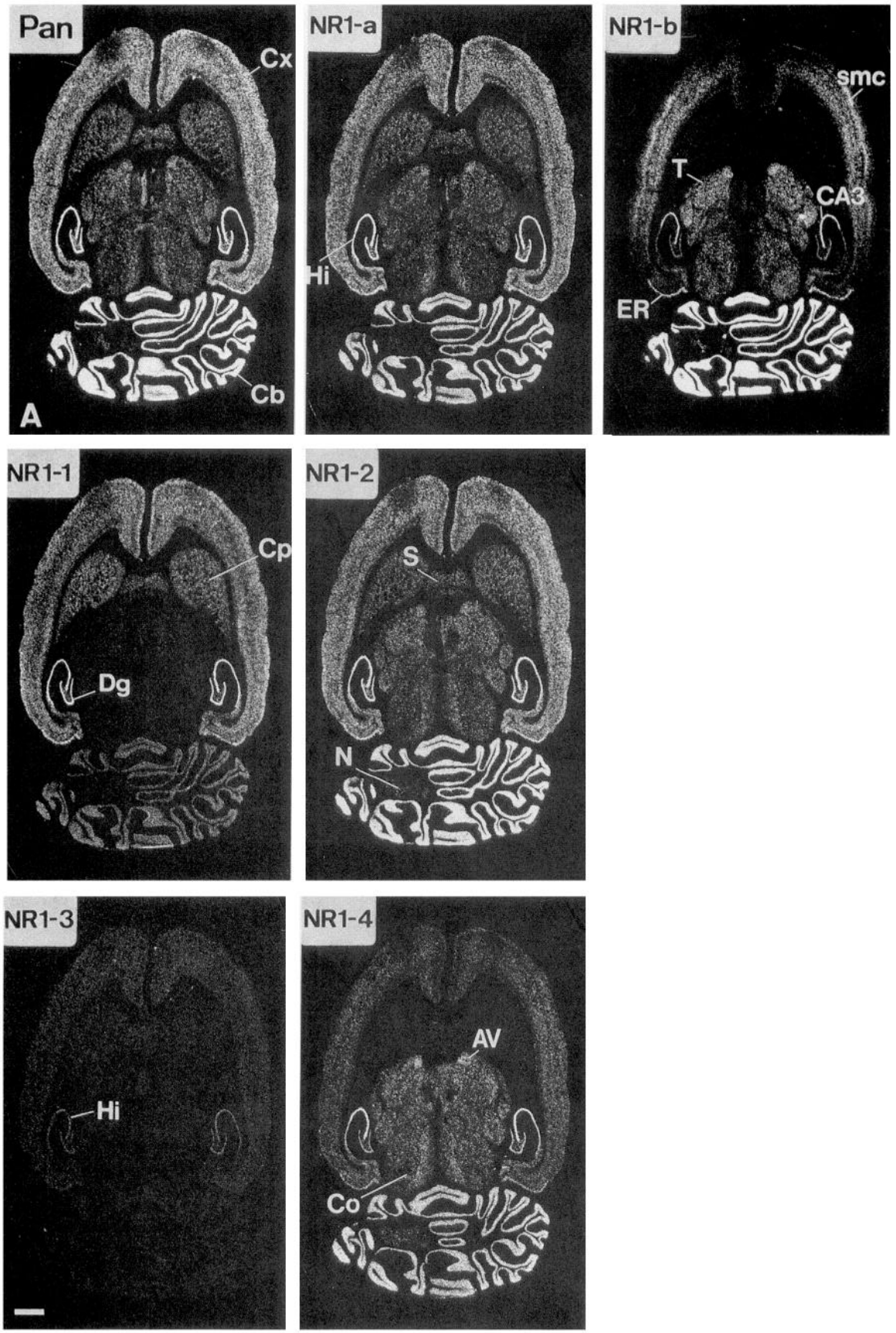

Figure 8. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to horizontal sections of adult rat brain. *Pan*, NR1-pan. For abbreviations, see Appendix. Scale bar, 1.8 mm.

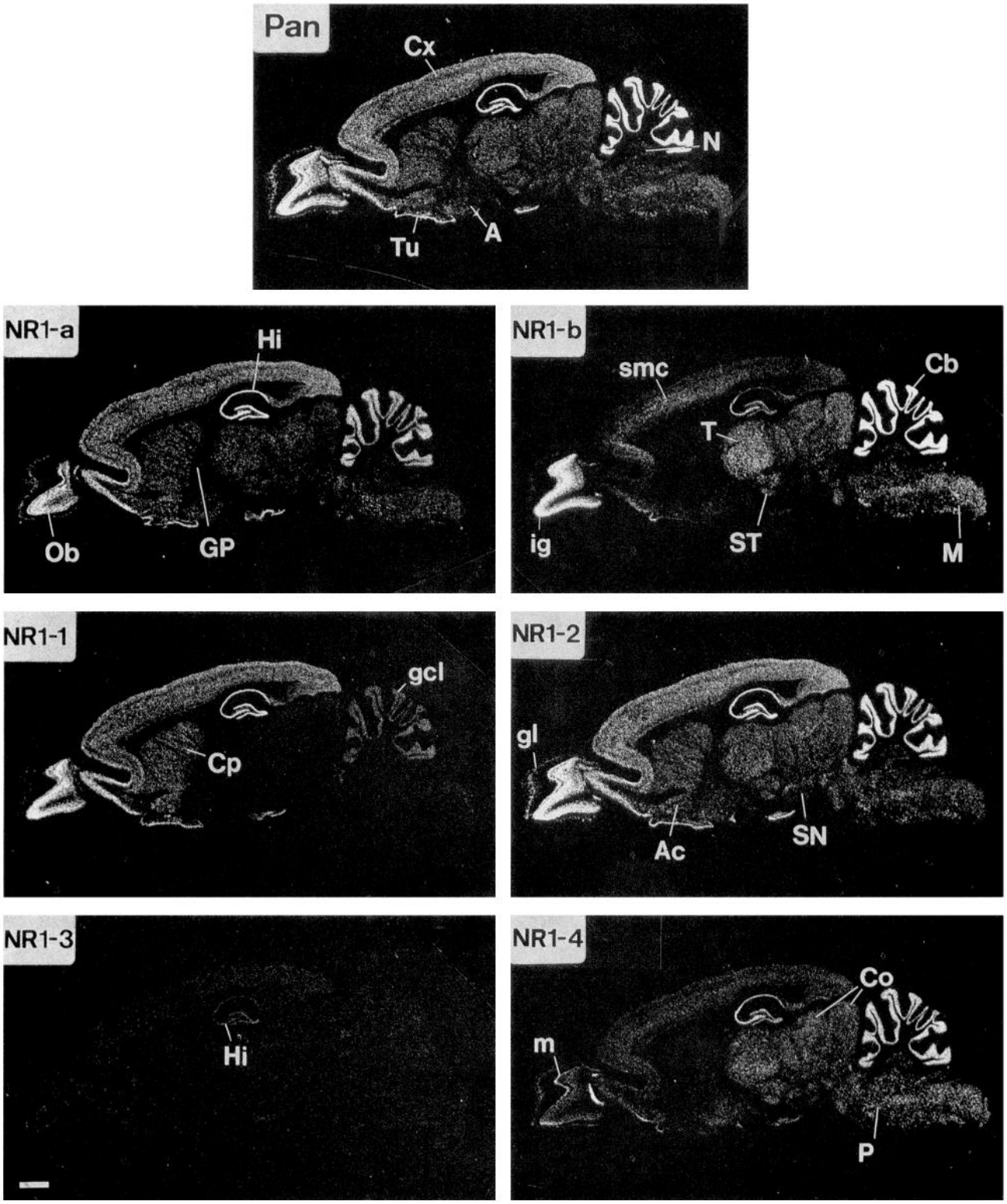

Figure 9. X-Ray film autoradiographs depicting the *in situ* hybridization of the NMDAR1 splice variant probes to sagittal sections of adult rat brain. *Pan*, NR1-pan. For abbreviations, see Appendix. Scale bar, 1.8 mm.

E17 and E19 (Figs. 3, 4). The most noticeable changes are the relatively greater proportion of NMDAR1-1 and -4 forms in mature, compared to perinatal, forebrain, and the change of dominant splice forms in the cerebellar granule cells (Figs. 5–10), which mature relatively late in development (Jacobson, 1991). Thus, throughout the postnatal development of each brain structure, similar populations of splice forms exist, each reaching a peak of abundance in the third postnatal week (Figs. 6–8). This peak, together with the concurrent peaks of expression of NMDAR2 subunit mRNAs (M. Watanabe et al., 1992; Mon-

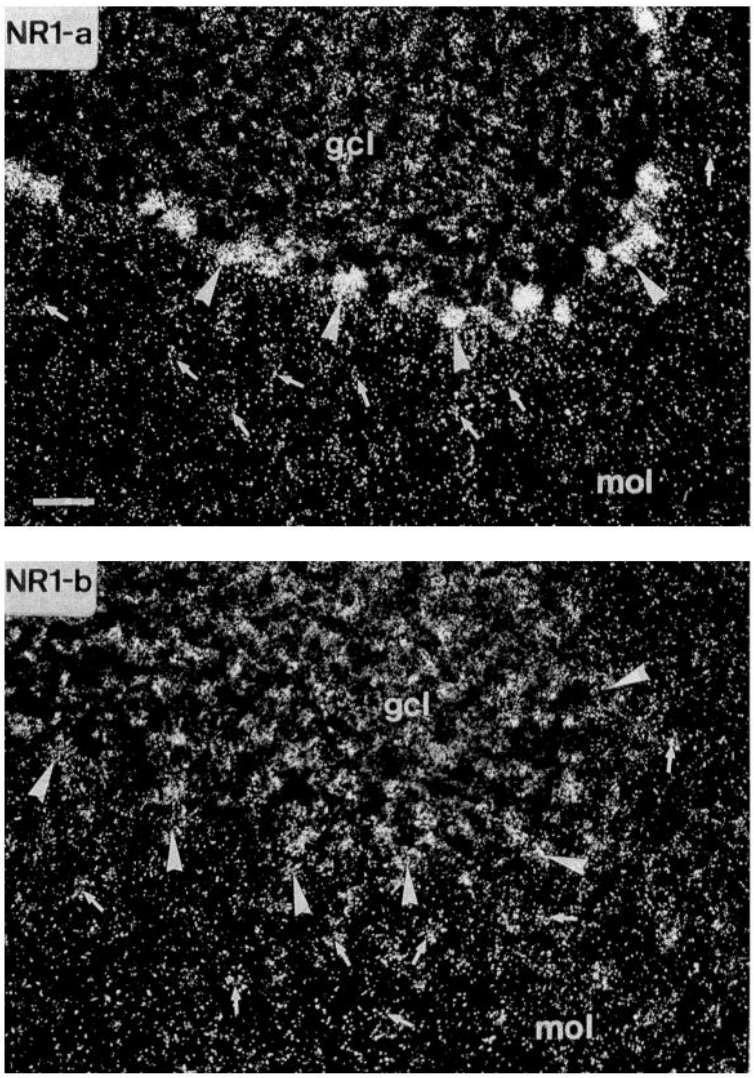

Figure 10. Dark-field photomicrographs depicting the *in situ* hybridization of two NMDAR1 splice variant probes (*NR1-a* and *-b*) to sagittal sections of adult rat cerebellum. *Arrowheads* indicate Purkinje cells; *arrows* indicate stellate-basket cells. For abbreviations, see Appendix. Scale bar, 50 μm.

yer, Burnashev, Laurie, Sakmann, and Seeburg, unpublished observations), coincides with the most active period of mature synapse formation (Aghajanian and Bloom, 1967). The inferred abundance and regional variety of NMDA receptors, consisting of different NMDAR1 splice variants and NMDAR2 subunits, would probably play an important role in regionally heterogeneous synaptic plasticity, synapse consolidation, and perhaps the subsequent elimination of excess neurons (McDonald and Johnston, 1990; Jacobson, 1991). Similar populations of splice forms appear to be retained at a lesser degree in structures of the mature brain, perhaps serving the same purpose of regionally heterogeneous synaptic plasticity (Collingridge and Singer, 1990). In the hippocampus, for example, the differential distribution of the NMDAR1-b and -4 variants, contrasting with the even signals using the other splice-specific probes (Figs. 8, 9), implies intrahippocampal heterogeneity of NMDAR1 splice variant populations. This molecular heterogeneity may be of physiological importance in the differences known to exist between Schaeffer collateral (CA1) and mossy fiber (CA3) types of synaptic potentiation (Staubli, 1992; Y. Watanabe et al., 1992; Zalutsky and Nicoll, 1992), or of pathological importance in the different sensitivities of the CA1 and CA3 regions to ischemic glutamate excitotoxicity (Mitani et al., 1992). Differential alternative splicing of the 5′ sequence of NMDAR1 mRNA often occurs in motion-associated structures (caudate (NMDAR1-a $\gg$ NMDAR1-b), P12 lateral caudate (a = b), sensorimotor cortex (a = b), thalamus (b > a), subthalamic nuclei (b > a), substantia nigra (a $\gg$ b), cerebellar Purkinje cells (a > b), cerebellar granule cells (b > a) (Figs. 7–10), suggesting that the two forms may have important physiological and perhaps pathological roles in motor coordination.

Functional heterogeneity

Some electrophysiological properties of the eight known NMDAR1 splice forms have been examined in oocytes by various groups. A consensus of the data indicates that relative to the NMDAR1-a variant, NMDAR1-b homomeric channels give greater maximum currents, and possess lower affinity for agonists but greater affinity for antagonists (both two- to fourfold) (Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992; Hollmann et al., 1993). These different ligand affinities have been proposed (Hollmann et al., 1993) as a possible basis for the heterogeneity of NMDA receptor glutamate binding sites found in rat brain (Monaghan et al., 1988). However, the affinity differences of the recombinant NMDAR1-a and -b homomeric assemblies for NMDA receptor antagonists are much smaller

than those differences observed in brain (20–50-fold) (Murphy et al., 1988; Sills et al., 1991). Additionally, although the NR1-1a and -b *in situ* hybridization images (Figs. 8, 9) are somewhat similar to the distribution of agonist- and antagonist-preferring binding sites, respectively (Monaghan et al., 1988; Monaghan, 1991; Monaghan and Beaton, 1992), there is considerable overlap of expression of the splice variants (Figs. 8, 9) and insufficient correlation with the ligand autoradiography to infer that the NMDAR1 5′ splice variant defines the ligand affinity. Recent experiments on recombinant heteromeric NMDAR1/NMDAR2 receptors show that large differences in agonist and antagonist affinity can be reproduced by changing the NMDAR2 subunit (Laurie and Seeburg, unpublished observations; D. T. Monaghan, personal communication). Moreover, the mRNA distributions for the NMDAR2 subunits (Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993) closely correlate to the distributions of the appropriate NMDA binding sites (Monaghan et al., 1988; Monaghan, 1991; Monaghan and Beaton, 1992). Whether alternative 5′ splice forms of the NMDAR1 mRNA in heteromeric recombinant NMDAR1/NMDAR2 assemblies can modulate ligand affinity, affect polyamine responses (Durand et al., 1992), or influence voltage dependency (Anantharam et al., 1992) remains to be tested.

Low concentrations of Zn^{2+} ions potentiate glutamate-gated currents at homomeric NMDAR1-a assemblies, but higher concentrations are inhibitory at both NMDAR1-a and -b homomeric receptors (Hollmann et al., 1993). The different distributions of NMDAR1-a and -b mRNAs (Figs. 8–10; Standaert et al., 1993) may not lead to regional heterogeneity in Zn^{2+} responses, however. Coexpression of NMDAR1-a and -b mRNA, a likely situation since NMDAR1-a mRNA seems to be present in every neuronal type, results in assemblies possessing only NMDAR1-a–like Zn^{2+} sensitivities (Hollmann et al., 1993). Moreover, the Zn^{2+} potentiation is lost in heteromeric NMDAR1-a/NMDAR2 assemblies (Hollmann et al., 1993), which is probably the dominant composition of cerebral NMDA receptors (Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993). Thus, the potentiation by Zn^{2+} may not occur, unless homomeric NMDAR1-a channels are formed by specific controlled assembly and/or by the absence of NMDAR2 subunits, such as appears to occur in adult cerebellar Purkinje cells (Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993), which also contain much more NMDAR1-a than -b mRNA (Fig. 10). The absence of NMDAR2 subunit mRNAs in adult cerebellar Purkinje cells, allowing only the production of poorly conducting homomeric NMDAR1 channels (Moriyoshi et al., 1991), may account for the lack of detectable NMDA-gated responses on these cells (Audinat et al., 1990; Hirano, 1990; Perkel et al., 1990; Farrant and Cull-Candy, 1991; Hussain et al., 1991).

Alternative splicing of the 3′ sequences of NMDAR1 mRNA does not appear to affect ligand affinities, responses to Zn^{2+} or polyamines, or sensitivity to Mg^{2+} inhibition (Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992; Yamazaki et al., 1992; Hollmann et al., 1993). Recently, recombinant homomeric NMDAR1-1a receptors were shown to be phosphorylated at the carboxy terminus to a much greater degree under baseline and experimental conditions than NMDAR1-2a assemblies, which hints to the carboxy terminus of the NMDAR1 subunit being located intracellularly (Tingley et al., 1993), in contrast to the extracellular location originally proposed (Moriyoshi et al., 1991). Use of consensus phosphorylation sites within the deletion cassettes, and their removal by alternative splicing, may regulate channel properties such as desensitization (Huganir and Greengard, 1990), or the induction of LTP by PKC (Ben-Ari et al., 1992). Indeed, NMDA-gated currents at recombinant homomeric NMDAR1-3b channels are much more sensitive to potentiation by PKC activators than those at NMDAR1-1a assemblies (Durand et al., 1992; Yamazaki et al., 1992), although receptors containing the NMDAR1-3b subunit are probably very scarce throughout development (Figs. 2–9). Regional and developmental regulation of splicing of the 3′ sequences of NMDAR1 mRNA, exemplified by the contrasting distributions of NMDAR1-1 and NMDAR1-4 mRNAs (Figs. 5–9), may control sensitivities to phosphorylation and therefore kinetic properties of the NMDA channels and their involvement in LTP and synaptic plasticity.

In conclusion, we have found that NMDAR1 mRNA expression develops in a caudal to rostral gradient, that markedly different but overlapping patterns of 5′ and 3′ splicing appear by birth, and that this regional heterogeneity is extensively maintained through the postnatal expression peak and subsequent decline to adult expression levels. Determination of the functional consequences of the regionally specific splicing is an intriguing prospect.

Appendix

List of abbreviations

5g	embryonic trigeminal ganglion
A	amygdala
Ac	nucleus accumbens
AV	anteroventral thalamic nuclei
CA3	hippocampal CA3 region
Cb	cerebellum
cb	embryonic cerebellum
cf	cephalic flexure
Co	colliculi
co	embryonic colliculi
Cp	caudate-putamen
cp	cortical plate
Cx	cortex
cx	embryonic cortex
Dg	dentate gyrus
di	embryonic diencephalon
drg	embryonic dorsal root ganglion
e	embryonic nasal epithelium
ER	entorhinal cortex
gcl	cerebellar granule cell layer
gl	glomerular layer
GP	globus pallidus
Hi	hippocampus
hi	embryonic hippocampus
hy	embryonic hypothalamus
ig	internal granule cell layer
L	embryonic liver
ls	lateral striatum
M	medulla
m	mitral cell layer
mes	embryonic mesencephalon
met	embryonic metencephalon
mol	cerebellar molecular layer
my	embryonic myelencephalon
N	deep cerebellar nucleus
Ob	olfactory bulb
ob	embryonic olfactory bulb
P	pons
p	embryonic pons
pt	embryonic pretectum
S	septum
sc	embryonic spinal cord
smc	sensorimotor cortex
SN	substantia nigra

ST	subthalamic nucleus
st	embryonic striatum
T	thalamus
t	embryonic thalamus
te	embryonic telencephalon
tg	embryonic tegmentum
Tu	olfactory tubercle

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