N-Methyl-D-Aspartate Receptor Subunit NR2B Is Widely Expressed Throughout the Rat Diencephalon: An Immunohistochemical Study

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N-Methyl-D-Aspartate Receptor Subunit NR2B Is Widely Expressed Throughout the Rat Diencephalon: An Immunohistochemical Study

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ABSTRACT
Glutamate (Glu), a major excitatory neurotransmitter within the hypothalamus and thalamus, acts upon many receptors, including the N-methyl-D-aspartate (NMDA) subtype. Abundant evidence suggests that variations in the subunit composition of NMDA receptors (NMDA-Rs) contribute to differences in Glu’s immediate electrophysiological effects as well as to the patterns of signal transduction cascades it triggers to mediate long-term changes in neuronal function. We have previously shown that hypothalamic NMDA-Rs containing the NR2B subunit may be involved in the control of eating as well as in the mediation of physiological responses to osmotic stimuli. To broaden our understanding of diencephalic NMDA-R participation in other functions, we localized the NR2B subunit in the diencephalon of the adult male rat using immunoperoxidase, immunogold, and immunofluorescence techniques and an affinity-purified polyclonal antibody specific for the NR2B subunit of the NMDA-R. In addition, we used a monoclonal NR2B antibody with immunoperoxidase detection to confirm the NR2B distribution seen with the polyclonal antibody. In the hypothalamus, the highest levels of NR2B immunoreactivity (-ir) were found in the magnocellular neurosecretory system, including the paraventricular and supraoptic nuclei. A new finding was that intense NR2B-ir was present within perivascular “accessory” magnocellular groups of this system, including the nucleus circularis, anterior fornical nucleus, and scattered clusters of lateral hypothalamic cells apposed to blood vessels. Robust NR2B-ir was also present within the arcuate nucleus, the median eminence, and the tuberal nucleus, and light immunostaining was found in all other hypothalamic nuclei examined. In the thalamus, the highest NR2B-ir was observed in the medial habenula and the anterodorsal, paraventricular, rhomboid, reticular, and dorsal lateral geniculate nuclei. As in the hypothalamus, all thalamic nuclei examined displayed at least light immunostaining for this subunit. Control sections, including those incubated with the polyclonal NR2B antibody preadsorbed with its fusion protein, were virtually devoid of immunostaining. This demonstration that the NR2B subunit of the NMDA-R is widely distributed in the diencephalon, implicates it in a wide variety of functions, and provides a useful anatomical framework for establishing a comprehensive map of Glu receptor populations within this major subdivision of the brain. J. Comp. Neurol. 428:428–449, 2000.

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Indexing terms: glutamate; hypothalamus; thalamus; epithalamus; magnocellular; neuroendocrine

In addition to the important role it plays in CNS intermediary metabolism (Strecker, 1957), the amino acid L-glutamate (Glu) is a key mediator of excitatory neurotransmission, transferring its chemical signal between neurons and/or glia (Gallo and Ghiani, 2000) by binding to ionotropic and metabotropic Glu receptors. To date, 17 ionotropic Glu receptor subunits have been cloned in mammals, six of which are classified as N-methyl-D-aspartate receptor (NMDA-R) subunits (Dingledine et al., 1999). These subunits (in the rat NR1, NR2A-D, NR3A)
are ontogenically expressed in highly regulated spatio-temporal patterns throughout the nervous system and, with the possible exception of NR3A (Villmann et al., 1999), assemble into functional heteromeric receptors with biochemical and electrical properties that vary with varying subunit composition (Vallano, 1998; Dingledine et al., 1999; Yamakura and Shimoji, 1999).

The NR2B subunit, the focus of the present study, has received much attention as a potential substrate and/or anchoring target for intracellular signalling molecules such as protein kinases and phospholipase Cγ (Moon et al., 1994; Lau and Huganir, 1995; Suzuki and Okumura-Noji, 1995; Omkumar et al., 1996; Gurd and Bisson, 1997; Strack and Colbran, 1998; Leonard et al., 1999) as well as for neuromodulators such as growth factors and hormones (Chen and Leonard, 1996; Lin et al., 1998; Christie et al., 1999). This subunit contains a Glu-binding site (Laube et al., 1997), has been implicated as a neural substrate underlying various forms of Glu-mediated synaptic plasticity (Rosenblum et al., 1995, 1996; Duda, 1996; Rostas et al., 1996; Tang et al., 1999), and also may play a role in the regulation of neurite outgrowth and pathfinding (Herbert et al., 1998). Moreover, some pathophysiological conditions are associated with alterations in the expression and posttranslational modification of the NR2B subunit (Akbarian et al., 1996; Arzberger et al., 1997; Takagi et al., 1997; Di Luca et al., 1999) or changes in the number of binding sites for NR2B-selective radioligands (Grimwood et al., 1999). Taken together, these facts highlight the important influence the NR2B subunit has on NMDA-R-mediated actions in the nervous system.

We are interested in determining the roles that hypothalamic NMDA-Rs and their subunits play in neuroendocrine function and in the control of food and fluid ingestion. We have recently shown that levels of NR1 and NR2B subunits within hypothalamic magnocellular nuclei are up-regulated and down-regulated, respectively, during dehydration (Decavel and Currás, 1997; Currás-Collazo and Dao, 1999) and that, in the hypothalamic supraoptic nucleus, both of these subunits exhibit plasticity during development (Currás and Dao, 1998). Moreover, pharmacological evidence indicates that the NR2A and/or NR2B subunits (Khan et al., 1999a) are components of functional NMDA-Rs mediating eating behavior in adult rats (Stanley, 1996; Stanley et al., 1996, 1997). In conjunction with reports documenting hypothalamic NMDA-R involvement in the control of circadian rhythms and the activity of magnocellular neuroendocrine cells (e.g., see Brann, 1995; Ebling, 1996; Rea, 1998; for reviews), these findings suggest NMDA-R subunit involvement in a variety of hypothalamic functions. The distribution of these subunits within certain diencephalic areas, however, remains poorly understood.

Recently, using a highly specific, affinity-puriﬁed polyclonal antibody raised against a portion of the C-terminal sequence of the NR2B subunit, we have immunohistochemically localized the NR2B subunit within the lateral hypothalamic area (Khan et al., 1999a), a brain region classically implicated in the control of eating and the regulation of body weight (Elmquist et al., 1999). Moreover, we have used this antibody to show that NR2B is present within vasopressin (VP)- and oxytocin (OT)-containing magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei (Currás-Collazo et al., 2000). We, along with others, have conﬁrmed the speciﬁcity of this antibody using both immunohistochemical (Bozzetti and Currás, 1998) and immunoblottting procedures (Snell et al., 1996; Currás and Dao, 1998; Currás-Collazo and Dao, 1999; Khan et al., 1999a).

To broaden our understanding of NR2B function, here we have extended our analysis of NR2B subunit localization to include the entire diencephalon by using this polyclonal antibody in immunoperoxidase, immunogold, and immunofluorescence studies. Additionally, we have used a monoclonal anti-NR2B antibody with immunoperoxidase detection in this study to conﬁrm the widespread diencephalic distribution of NR2B that we observed using the polyclonal antibody. Our data, portions of which have been presented together by our laboratories in preliminary form (Bozzetti and Currás, 1998; Khan et al., 1998), suggest that the NR2B subunit is a likely participant in a wide array of processes subserved by functionally active diencephalic NMDA-Rs.

**MATERIALS AND METHODS**

**Materials**

We used two different primary NR2B antibodies: 1) an affinity-puriﬁed rabbit anti-rat antibody targeted against

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
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<tbody>
<tr>
<td>3V</td>
<td>third ventricle</td>
</tr>
<tr>
<td>AD</td>
<td>anterodorsal nucleus thalamus</td>
</tr>
<tr>
<td>AHA</td>
<td>anterior hypothalamic area</td>
</tr>
<tr>
<td>ARH</td>
<td>arcuate nucleus hypothalamicus</td>
</tr>
<tr>
<td>AV</td>
<td>anteroventral nucleus thalamus</td>
</tr>
<tr>
<td>dLGN</td>
<td>lateral geniculate nucleus thalamus, dorsal region</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial nucleus hypothalamicus</td>
</tr>
<tr>
<td>fr</td>
<td>fasciculus retroflexus</td>
</tr>
<tr>
<td>fx</td>
<td>columns of the fornix</td>
</tr>
<tr>
<td>ic</td>
<td>internal capsule</td>
</tr>
<tr>
<td>LGN</td>
<td>lateral geniculate nucleus thalamus</td>
</tr>
<tr>
<td>LHA</td>
<td>lateral hypothalamic area</td>
</tr>
<tr>
<td>LHPN</td>
<td>lateral hypothalamic perivascular nuclei</td>
</tr>
<tr>
<td>LPO</td>
<td>lateral preoptic area</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
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<tr>
<td>MH</td>
<td>medial habenula</td>
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<tr>
<td>MPO</td>
<td>medial preoptic area</td>
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<tr>
<td>NC</td>
<td>nucleus circularis</td>
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<td>oc</td>
<td>optic chiasm</td>
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<tr>
<td>ot</td>
<td>optic tract</td>
</tr>
<tr>
<td>PT</td>
<td>parataenial nucleus thalamus</td>
</tr>
<tr>
<td>PVa</td>
<td>anterior periventricular nucleus hypothalamicus</td>
</tr>
<tr>
<td>PVH</td>
<td>paraventricular nucleus hypothalamicus</td>
</tr>
<tr>
<td>PVT</td>
<td>paraventricular nucleus thalamus</td>
</tr>
<tr>
<td>RE</td>
<td>reuniens nucleus thalamus</td>
</tr>
<tr>
<td>RH</td>
<td>rhomboid nucleus thalamus</td>
</tr>
<tr>
<td>RTN</td>
<td>reticular nucleus thalamus</td>
</tr>
<tr>
<td>SCH</td>
<td>suprachiasmatic nucleus hypothalamicus</td>
</tr>
<tr>
<td>SUB</td>
<td>submedial nucleus thalamus</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus hypothalamicus</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>VMH</td>
<td>ventromedial nucleus hypothalamicus</td>
</tr>
<tr>
<td>VPN</td>
<td>ventral posterior nucleus thalamus</td>
</tr>
<tr>
<td>VPI</td>
<td>ventral postero lateral nucleus thalamus</td>
</tr>
<tr>
<td>VPm</td>
<td>ventral postero medial nucleus thalamus</td>
</tr>
<tr>
<td>XI</td>
<td>xiphoid thalamic nucleus</td>
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the amino acid sequence at positions 984–1,104 of the NR2B subunit C-terminus (purchased from Chemicon International, Temecula, CA, or Calbiochem, San Diego, CA) and 2) a monoclonal antibody raised against the antigen used by Wang et al., 1995 (generously provided by Dr. Barry Wolfe). Biotinylated goat anti-rabbit immunoglobulin G (IgG; heavy + light chains) and the avidin-biotin-peroxidase (ABC Elite Kit) solution were from Vector Laboratories (Burlingame, CA), as was the biotinylated horse anti-mouse IgG. Goat anti-rabbit IgG conjugated with 1 nm gold particles was purchased from BN International (Cardiff, United Kingdom). Molecular Probes (Eugene, OR) supplied the goat anti-rabbit Oregon Green secondary antibody. All other materials were purchased from Sigma Chemical Co. (St. Louis, MO), Vector Laboratories, or local suppliers. Percentage concentrations are expressed in volume percentage unless otherwise noted, in which case, the notation is made only at the first mention of the solute.

Methods

Procedures were carried out in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and were approved by the University of California at Riverside Chancellor’s Committee on Laboratory Animal Care. Care was taken to avoid animal suffering.

Note on detection methods

The immunohistochemical detection of the NR2B subunit was performed using three types of immunoprobes (peroxidase, silver-intensified gold, fluorescent) to eliminate the potential artifacts that can be produced with any one method and to take advantage of the visualization properties unique to each. For example, immunoperoxidase (IP) detection using 3,3′-diaminobenzidine (DAB) tetrahydrochloride is very sensitive and can detect labeling in areas of light protein expression. However, it can also intensely label intracellular organelles such as peroxisomes (see Peters et al., 1991, for references), thus potentially staining cells that may not contain NR2B antigen, an artifact not found with the other techniques. DAB may also precipitate on cellular surfaces that are near the peroxidase enzyme but not necessarily immunohistochemically linked to it (van den Pol, 1989). The IG visualization method is a reliable alternative to IP labeling that still provides a stable, virtually permanent retention of immunolabel in cells. This is in contrast to some immunofluorescent (IF) signals, which are labile. The IP method, however, can be used with confocal microscopy, which often provides better anatomical resolution of immunoreactive cells and fibers. Another advantage of the IF, as well as the IG, methods we employed is that biotinylated antibodies were not used, eliminating the possibility that endogenous biotin might have accounted for some of the labeling that can be produced when using biotinylated compounds, a problem recently reported by Hymes and Wolf (2000). Labeling of endogenous biotin is also unlikely to have contributed to our results, because labeling was absent in our sections processed with biotinylated compounds but without the primary antibody and because endogenous biotin was reportedly absent in examined portions of the rat diencephalon (Wang and Pevsner, 1999).

Tissue preparation

As described previously (Khan et al., 1999a), adult male Sprague-Dawley or Holtzman rats (325–475 g; n = 57) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the ascending aorta with at least 100 ml of cold, heparinized phosphate-buffered saline [0.01 M sodium phosphate buffer in 0.9% (w/v) saline (PBS), containing 200 units of heparin (liter), followed by at least 300 ml of ice-cold 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brain was quickly dissected, and a block of diencephalic tissue was removed (see below under Anatomical delimitations, nomenclature, and analysis of tissue). For tissue to be sectioned on a cryostat, postfixation using the same fixative (immersion at 4°C for at least 24 hours) was performed. Postfixed blocks were then transferred to a 25% (w/v) sucrose cryoprotectant solution at 4°C for at least 24 hours, quick frozen in dry ice, sectioned on a Leica cryostat into 25-μm-thick coronal sections, and collected in cold PBS. Other tissue blocks were sectioned on a vibratome (50–70-μm-thick; coronal plane) and collected in cold PBS.

For IP experiments only, free-floating sections were reacted with 0.1–0.5% (w/v) sodium borohydride for 10 minutes at room temperature (RT), followed by 0.1% hydrogen peroxide in PBS for 10 minutes at RT, and then rinsed for 10 minutes in PBS prior to the blocking step. Blocking involved incubating the sections for 1 hour at RT in 5–10% normal goat serum (NGS) or normal horse serum (NHS) and 1% (w/v) bovine serum albumin (BSA) in 0.3% Triton X-100 containing PBS (T-PBS). In some experiments, 0.02% (w/v) sodium azide (NaN3) was included in the blocking solution.

Immunodetection

Primary antibody reactions. For IP experiments, sections were incubated in one of two ways: 1) with the rabbit anti-rat NR2B antibody (1:600–1:1,000) in 0.3–0.5% T-PBS containing 1% NGS, 0.2% BSA, and 0.02% NaN3 for ~72 hours at 4°C; or 2) with the mouse monoclonal NR2B antibody (1:500) in 0.1% T-PBS containing 1% NHS, 0.2% BSA, and 0.02% NaN3 for 40 hours at 4°C. For IG experiments, only the polyclonal NR2B antibody was used at 1:600–1:1,000 in 0.01 M PBS containing 1% NGS and 0.2% BSA for 36–72 hours at 4°C. For IF experiments, the polyclonal NR2B antibody was diluted (1:500) in either PBS, pH 7.4, or Tris-buffered saline (TBS; 0.01 M, pH 7.6, 1.2% NaCl) containing 0.02% NaN3, and incubated for not more than 40 hours at 4°C. All sections were then rinsed three or four times with chilled T-PBS for at least 10 minutes each time.

IP experiments. Sections were reacted with either a biotinylated goat anti-rabbit IgG or a biotinylated horse anti-mouse IgG. In either case, the secondary antibody was diluted 1:200 in 0.1% T-PBS for 1 hour at RT, and sections were then incubated for 1 hour at RT in avidin-biotin-peroxidase complex (ABC) solution in T-PBS (1:150). They were then transferred briefly to a 0.05 M Tris HCl solution containing 0.9% NaCl and preincubated in a DAB chromogen solution (20 mg DAB/100 ml of Tris HCl) for 5–10 minutes at RT, before being reacted with 1–4 μl of 30% H2O2 in 10 ml DAB solution. NiSO4 (0.15 g/100 ml DAB solution) was sometimes added to intensify the stain, and reactions were stopped with cold Tris HCl.
**IG experiments.** Sections were reacted with a goat anti-rabbit IgG conjugated with 1 nm gold for 1.5 hours at RT. This conjugate was diluted (1:20–1:60) in 0.01 M PBS containing 0.1% (w/v) gelatin and 0.4% (w/v) L-lysine. Silver intensification of the gold particles was performed using a protocol modified from van den Pol and Decavel (1990) and as described previously (Decavel and Currás, 1997). This procedure yielded dark brown or gray punctate labeling over cells containing NR2B-ir when observed under brightfield optics.

**IF experiments.** Sections were rinsed several times in PBS or TBS and then incubated for 1 hour at RT with a goat anti-rabbit antibody conjugated to Oregon green (1:800), which was diluted in 0.1 M TBS. This procedure yielded a green signal over cells containing NR2B-ir under epifluorescent optics.

**Tissue mounting and observation**

Sections were mounted onto gelatin/chromate-subbed slides and air dried. For IP and IG studies, sections were then dehydrated and in some cases counterstained with thionin or hematoxylin. For IF experiments, sections were rinsed in water. All slides were then coverslipped, by using either DPX (IP, IG labels) or Vectashield (IF label). Brain regions were then examined and photographed with a Nikon Microphot FX-A light microscope under either brightfield illumination or epifluorescence using appropriate filters. IF-labeled sections were also examined using a Zeiss laser confocal system (LSM 510) equipped with a Zeiss Axioplan upright microscope and an argon/helium-neon laser combination. Images were obtained as single confocal images using a 40× oil immersion objective and appropriate filters for the detection of Oregon Green. Images, 512 × 512 pixels in size and with an eight-bit pixel depth, were obtained and averaged using Kalman filtering (four images) to reduce noise. Confocal images were reconstructed in color, cropped to size, contrast-enhanced using Adobe Photoshop 5.0 software, and printed on a Sony UP-D1500CN dye sublimation printer. For IP and IG images, photomicrographs were prepared using either Kodak Royal Gold film (ASA 25) or Kodak Technical Pan film 5612 (ASA 25).

**Immunohistochemical controls**

A subset of brain sections in all experiments was treated identically, except that a solution containing PBS (with or without Triton X-100/serum/BSA) was substituted for each primary antibody. In other control experiments, the secondary antibody was also replaced with T-PBS. To test further the specificity of NR2B-ir, in other studies the polyclonal NR2B antibody was preadsorbed with its fusion protein used to immunize rabbits. The NR2B fusion protein was synthesized using the deduced amino acid sequence at positions 984–1,104 of the putative C-terminal region of this subunit (Ishii et al., 1993). NR2B labeling was virtually absent in brain sections incubated with the polyclonal antibody preadsorbed with a 20-fold excess of fusion protein at 4°C for 24 hours (see Results).

**Anatomical delimitations, nomenclature, and analysis of tissue**

**Rostrocaudal extent of analyzed tissue.** Figure 1 depicts the approximate rostrocaudal limits of the brain block that was analyzed. Sections throughout the diencephalon, except for a few portions of the posterior thalamus and anterior hypothalamus, were collected (see legend to Fig. 1).

**Nomenclature.** In this study, the “diencephalon” is defined as outlined in the rat brain stereotaxic atlas by Swanson (1998). Thalamic divisions include the epithalamic, dorsal thalamus, and ventral thalamus (the latter including the zona incerta). The major hypothalamic cell groups (see Clark, 1938; Crosby and Woodburne, 1940; Swanson 1983) fall within three longitudinal zones (periventricular, medial, lateral) and four rostrocaudal divisions (preoptic, anterior, tuberal, mammillary). Because of its well-established functional importance, the hypothalamic magnocellular neurosecretory system is described separately. Although this system includes cells scattered in many areas (reviewed by Swanson, 1983; see
especially Fig. 3 of this review), our use of “magnocellular neurosecretory system” is limited to the paraventricular, supraoptic, and perivascular “accessory” magnocellular hypothalamic nuclei. The NR2B-ir seen in portions of these nuclei is putatively assigned to “neurosecretory” cells on the basis of the well-documented cytoarchitectural, chemoarchitectural, and projection profiles of these cells (Peterson, 1966; Sherlock et al., 1975; Fisher et al., 1979; Ju et al., 1986) as well as on our dual-label immunohistochemical data showing colocalization of NR2B with OT- or VP-neurophysin immunoreactivity (Bozzetti and Curra’s, 1998; Curra’s-Collazo et al., 2000).

The subthalamic nucleus is included as a hypothalamic structure, after Swanson (1998), although it is frequently included as a functional component of the basal ganglia (e.g., see Wilson, 1990). The thalamic paraventricular nucleus (PVN) is considered here as part of the epithalamus and not the midline thalamic nuclei, in keeping with Jones (1985). Names and parcellation of all other cell groups and areas within the diencephalon generally follow those in the atlas of Swanson (1998), with the following exceptions.

Midline group of the dorsal thalamus. Following Paxinos and Watson (1998), we include the xiphoid thalamic nucleus as a midline nucleus.

Perivascular “accessory” neuronal groups of the hypothalamic magnocellular neurosecretory system. These scattered magnocellular nuclei, first characterized by Peterson (1966), are closely apposed to blood vessels (Ambach and Palkovits, 1979) and have been called “perivascular neuronal groups” (Duan and Ju, 1998). Such groups that lie just dorsal to the optic tract have been collectively designated as the “lateral hypothalamic perivascular nucleus” (LHPN). Additionally, NR2B-labeled cells are likely branches of either the supraoptic, and perivascular “accessory” magnocellular hypothalamic nuclei. The NR2B-ir seen in portions of these nuclei is putatively assigned to “neurosecretory” cells, and not the midline thalamic nuclei, in keeping with Jones (1985). Names and parcellation of all other cell groups and areas within the diencephalon generally follow those in the atlas of Swanson (1998), with the following exceptions.

RESULTS

Immunohistochemical controls

Omitting the primary antibody always virtually eliminated labeling, leaving only weak, nonspecific staining across techniques. This is shown for IP detection in Figure 2B. Omitting the secondary antibody also eliminated staining (data not shown). Further evidence for the specificity of NR2B-ir is that brain sections incubated with the polyclonal NR2B antibody, preadsorbed with its fusion protein, showed virtually no labeling. This can be seen for the SON in Figure 3A,C.

General staining pattern

The pattern of NR2B labeling was nearly identical across all three techniques. The intensity of NR2B-ir was comparable between tissue processed using IP and IG detection but was sometimes less with IF visualization, except in magnocellular cells (see footnote 2 to Table 1). As is summarized in Table 1, all examined hypothalamic and thalamic structures displayed at least light NR2B-ir, which is similar to reports of widespread NR2B mRNA expression throughout the forebrain (Watanabe et al., 1993). Immunostaining was observed in neuronal cell bodies as well as in the neuropil. Finally, the patterns of staining produced by the monoclonal and polyclonal antibodies were essentially identical (see footnote 1 to Table 1). Collectively, these data provide converging evidence that the reported NR2B-ir is due to the presence of authentic NR2B subunits.

In a few experiments, occasionally including “no primary antibody” control experiments, the ependymal cell layers lining the third ventricle were also immunostained, both at the level of the hypothalamus and, more dorsally, at the level of the medial habenula. Thus, this immunostaining is unlikely to have been due to specific labeling of the NR2B subunit.

NR2B-ir in the hypothalamus

Magnocellular neurosecretory system

Paraventricular hypothalamic nucleus (PVH). Magnocellular neurosecretory cells of this nucleus displayed strong NR2B-ir (Table 1, Fig. 2A), among the highest found in our analysis of the diencephalon. This was true for the magnocellular PVH even when viewed at high magnification, when the high cell packing density of the nucleus did not bias our analysis. These cells were more heavily and uniformly labeled than parvocellular cells of this nucleus (Fig. 2A). Viewed at high magnification, staining was observed in perikarya and neuropil.

Supraoptic nucleus (SON). Intense NR2B-ir was uniformly distributed throughout cells in this nucleus, suggesting the presence of this subunit in cells containing OT as well as in those containing VP (Figs. 2C, 3A). We have confirmed this in dual-label experiments colocalizing the NR2B subunit with either OT- or VP-neurophysin (Curra’s-Collazo et al., 2000). Strong labeling was observed in magnocellular perikarya, particularly in ventrally located SON cells, and moderate NR2B-ir was also observed in the dendritic zone just dorsal to the ventral glia limitans, an area where dendrites extending from SON somata are known to be prevalent. Posteriorly in the tuberal hypothalamus, the retrochiasmatic portion of the SON also contained moderately to strongly labeled NR2B-immunoreactive cells.

Perivascular magnocellular nuclei. Strong immunostaining was observed in cells of the anterior fornical nucleus (AFN; Fig. 2E) and nucleus circularis (NC; Fig. 2F) and in scattered nuclei within the lateral zone of the hypothalamus, collectively called the “lateral hypothalamic perivascular nucleus” (LHPN). Additionally, NR2B-immunoreactive cells were observed scattered in areas dorsal to the SON and the optic tract. These magnocellular cells, which were found along blood vessels, displayed prominent immunostaining, as represented in Figure 2D. To our knowledge, these specific cells have not been identified in studies of the perivascular magnocellular nuclei (Peterson, 1966; Duan and Ju, 1998), although they probably can be placed within the cell groups of the LHPN. Judging by the rostrocaudal placement of the sections containing these cells, the blood vessels apposed to these NR2B-labeled cells are likely branches of either the suprachiasmatic or the lateral hypothalamic arteries, most probably the latter (Ambach and Palkovits, 1979).
Fig. 2. NR2B-ir in selected hypothalamic magnocellular neurosecretory groups using the polyclonal NR2B antibody. A,B: The hypothalamic paraventricular nucleus either immunoreacted with (A) or without (B) the primary antibody. Care was taken to ensure that the photographic development and exposure times were similar for the immunopositive image and the control image. Note the robust presence of NR2B-ir in magnocellular PVH cells (arrows in A). C: Silver-intensified gold-labeled NR2B-ir in the supraoptic nucleus (SON). D: NR2B-ir cells in a more posterior portion of the supraoptic nucleus and in perivascular magnocellular cells within the field of the lateral hypothalamic area (LHA); IP technique. Asterisks, blood vessels; arrows, immunolabeled perivascular cells (thionin counterstain). E: Robust NR2B-ir in the anterior fornical nucleus (AFN); IP technique. F: Robust NR2B-ir in nucleus circularis (NC); IP technique. Scale bars = 100 μm in A,B,D; 50 μm in C,F; 25 μm in E.
Fig. 3. Specificity of NR2B-ir (A,C) and profiles of selected NR2B-positive cells of the hypothalamic periventricular zone (B,D) using the polyclonal NR2B antibody. A: IP-labeled cells of the supraoptic nucleus immunoreactive for NR2B (brown) and counterstained with thionin (blue). C: Control section containing the supraoptic nucleus, comparable to that shown in A (thionin counterstain). This section was incubated with the polyclonal NR2B antibody, preadsorbed with its fusion protein, which eliminated NR2B-ir. B: Periventricular IP-labeled neurons positive for the NR2B subunit (thionin counterstain). D: IF-labeled NR2B-ir cells of the arcuate nucleus. E: Map showing the field in D (rectangle) as well as surrounding brain areas. (This map was modified from Swanson LW [1998/1999] Brain maps: structure of the rat brain, 2nd ed. Amsterdam: Elsevier [CD-ROM graphic files].) Coronal plane. Scale bars = 50 μm in A,C,D; 25 μm in B.
Periventricular zone

Periventricular cell groups. All three chromogens revealed NR2B-immunoreactive periventricular cells at the rostrocaudal level of the preoptic hypothalamus. In particular, prominent NR2B-ir was found in cells of the periventricular part of the PVH, only some of which were magnocellular, and in cells bordering the middle portion of the third ventricle, most probably part of the anterior periventricular nucleus (Fig. 3B). This labeling was observed in the perikarya, in the neuropil, and in the subependymal layer, which has been shown by others to contain dendrites from magnocellular neuroendocrine cells (van den Pol, 1982).

Dorsomedial hypothalamic (DMH) and arcuate (ARH) nuclei, median eminence (ME). Cells in the DMH displayed light to moderate NR2B-ir (Table 1), whereas cells in the ARH were moderately to intensely immunostained (Fig. 3D). IP labeling in the ARH was more pronounced in cells flanking the ventral portion of the third ventricle than in cells directly ventral to the lumen's nadi (not shown). The internal layer of the ME also displayed moderate to fairly robust labeling (Table 1).

Medial zone

Medial preoptic (MPO) and anterior hypothalamic (AHA) areas; suprachiasmatic nucleus (SCH). Generally, cells within both MPO and AHA displayed light to moderate NR2B-ir (Table 1). NR2B-ir in the AHA did not lend itself to discrete clustering at any rostrocaudal level, except at the level including the SCH. In the SCH, NR2B-ir was moderate and uniformly distributed in the perikarya and neuropil (Fig. 4A). Cells of the reticulohypothalamic tract were also moderately immunostained for the NR2B subunit.

Ventralmedial nucleus (VMH) and posteromedial hypothalamic. At the level of the posterior portion of the PVH (level 27 in Swanson, 1998), NR2B-ir was moderate in cells of the VMH. In this nucleus, the distribution of NR2B-ir was widespread and fairly even, with minimal clustering. Also, a fairly uniform continuum of NR2B-ir cells was observed from this nucleus to the adjacent lateral hypothalamic area (LHA). Cellular and neuropil staining in the posterior hypothalamic nucleus were also light to moderate (Table 1).

Lateral zone

Lateral preoptic (LPO) and lateral hypothalamic (LHA) areas; tuberal and subthalamic nuclei. Many lightly to moderately NR2B-ir cells were found throughout the LPO (Table 1). Figure 4B shows NR2B-ir in cells of the LHA. Strong NR2B-ir was especially present in cells clustered near and around the columns of the fornix (perifornical region). Labeling could be seen in perikarya, and NR2B-ir in the neuropil was moderate. The strongest NR2B-ir in the LHA was found in the tuberal nucleus at a rostrocaudal level that included the ARH (Table 1). Labeling was moderate to fairly even, with minimal clustering. Also, a fairly uniform continuum of NR2B-ir cells was observed from this nucleus to the adjacent lateral hypothalamic area (LHA). Cellular and neuropil staining in the posterior hypothalamic nucleus were also light to moderate (Table 1).

NR2B subunit. The NR2B subunit, also known as NR2B or NR2B2, is a member of the NMDA receptor family and is expressed in a variety of brain areas. The NR2B subunit plays a critical role in neuronal function, particularly in synaptic plasticity and learning and memory. The NR2B subunit is expressed in high levels in the hypothalamus, which is involved in the regulation of various physiological functions such as body temperature, appetite, and circadian rhythms.

Table 1: Distribution of NR2B NMDA Receptor Subunit Immunoreactivity

<table>
<thead>
<tr>
<th>Hypothalamus</th>
<th>Thalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity²</td>
<td>Intensity³</td>
</tr>
<tr>
<td>Periventricular zone</td>
<td></td>
</tr>
<tr>
<td>Paraventricular n.</td>
<td></td>
</tr>
<tr>
<td>Magnocellular division†</td>
<td>3</td>
</tr>
<tr>
<td>Farvocellular division†</td>
<td>2–2.5</td>
</tr>
<tr>
<td>Periventricular part**</td>
<td>2</td>
</tr>
<tr>
<td>Dorsomedial n. hypothalamus</td>
<td>1–2</td>
</tr>
<tr>
<td>Arcuate n. hypothalamus†</td>
<td>2–3</td>
</tr>
<tr>
<td>Median eminence (internal layer)†</td>
<td>2–2.5</td>
</tr>
<tr>
<td>Paraventricular n.</td>
<td></td>
</tr>
<tr>
<td>Magnocellular division†</td>
<td>3</td>
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<tr>
<td>Farvocellular division†</td>
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<tr>
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<td>Dorsomedial n. hypothalamus</td>
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<tr>
<td>Median eminence (internal layer)†</td>
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</tr>
<tr>
<td>Medial zone</td>
<td></td>
</tr>
<tr>
<td>Median preoptic area</td>
<td>1–2</td>
</tr>
<tr>
<td>Median preoptic n.</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Suprachiasmatic n.†</td>
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</tr>
<tr>
<td>Retrorhachiasmatic area</td>
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<tr>
<td>Perivascular magnocellular n.</td>
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<tr>
<td>n. circularis*</td>
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<tr>
<td>Tuberal area</td>
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<td>Posterior n.</td>
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<tr>
<td>Lateral hypothalamic area</td>
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<tr>
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<tr>
<td>Supraoptic n.†</td>
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<td>Lateral hyp. perivascular n.†</td>
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<tr>
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<tr>
<td>Lateral thalamus</td>
<td>2–3</td>
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<tr>
<td>Median eminence</td>
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<tr>
<td>Intralaminar nuclei</td>
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<td>Central medial n.</td>
<td>2</td>
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<tr>
<td>Lateral group</td>
<td>2</td>
</tr>
<tr>
<td>Lateral posterior n.</td>
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</tr>
<tr>
<td>Ventral group</td>
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<tr>
<td>Ventromedial n.</td>
<td>2</td>
</tr>
<tr>
<td>Ventral posteromedial n.</td>
<td>2</td>
</tr>
<tr>
<td>Ventral posterolateral n.</td>
<td>2</td>
</tr>
<tr>
<td>Geniculate group</td>
<td>2</td>
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<tr>
<td>Dorsal part, lateral geniculate n.</td>
<td>2</td>
</tr>
<tr>
<td>Ventral thalamus</td>
<td>2</td>
</tr>
<tr>
<td>Centrolateral n.</td>
<td>2</td>
</tr>
<tr>
<td>Zona incerta</td>
<td>2–3</td>
</tr>
</tbody>
</table>

¹Note that some hypothalamic and thalamic structures were not retained in the blocks of tissue analyzed and are therefore not shown in this table. Please refer to Figure 1 for details. The NR2B-ir for those sites marked by an asterisk is reported based on experiments using only the polyclonal NR2B antibody; all unmarked sites are reported based on experiments using both the monoclonal and the polyclonal antibodies.

²To score the intensity of NR2B-ir, each NR2B-positive brain region was compared to all other NR2B-positive brain regions within tissue sections sampled from a single experiment.

³Contrary to the arrangement of Swanson (1998), which is generally followed here, the SON is considered as a nucleus of the lateral zone and the paraventricular thalamic nucleus as a portion of the epithalamus.
strongest in more medially located cells. Cells of the subthalamic nucleus were moderately immunopositive for the NR2B subunit. The intensity of staining was higher in this nucleus than that of the adjacent zona incerta, which is described below.

NR2B-ir in the thalamus

Epithalamus. Moderate to strong NR2B labeling was found in cells of the medial habenula (MH; Fig. 5A, Table 1). This NR2B-ir was concentrated in cells near the dorsal third ventricle and extended to the stria medullaris and fasciculus retroflexus. In contrast, cells of the lateral habenular nucleus were lightly to moderately immunostained (not shown). The paraventricular thalamic nucleus (PVT) displayed moderate to fairly robust NR2B-ir. Somata of the PVT were heavily labeled (Fig. 5C); this was largely confined to the region near the dorsal third ventricle, although the region just ventral to the nadir of the ventricular border displayed little to no immunoreactivity (not shown). In contrast, prominent labeling was observed in symmetrically descending mediolateral gradients on either side of this unlabeled region of the nucleus. Some of this labeling appeared to cross into the mediodorsal nuclei.

Dorsal thalamic nuclei

Anterior nuclear group. The strongest NR2B-ir for nuclei among this group was found in cells of the anterodorsal (AD) nucleus (Fig. 6E, Table 1). In contrast, neurons of the anteroventral (AV) nucleus displayed more moderate NR2B-ir (Fig. 6E). As has been noted by others (Jones, 1985), we observed that AD cells were larger and more intensely stained than cells of the AV nucleus, and a clear boundary could be readily observed between these nuclei, especially under epifluorescence (Fig. 6E). Cells of the anteromedial, mediodorsal, and laterodorsal nuclei displayed light to moderate NR2B-ir (Table 1). Moderate to fairly robust labeling was found in the ventral posterior lateral complex (VL) (Fig. 6C).

Midline nuclear group. Light to moderate labeling was found in the nucleus reuniens (RE) and moderate labeling was found in the smaller, more compact xiphoid (XI) thalamic nucleus that bisects the RE along the coronal plane (Fig. 5C, Table 1). Moderate to fairly robust labeling was found in the parataenial (PT) nucleus (Fig. 6C).

Intralaminar thalamic nuclei. Moderate to strong NR2B-ir was found in the rhomboid (RH) thalamic nucleus (Fig. 5C). Labeling was uniformly distributed within this region, as well as within the central medial thalamic nucleus; this latter nucleus, however, was only lightly immunopositive for NR2B.

Lateral, ventral and geniculate nuclear groups of the dorsal thalamus. Light to moderate NR2B-ir was observed in cells of the lateroposterior nucleus as well as the ventral anterior-lateral complex and the ventromedial, ventral posteromedial, and ventral posterolateral nuclei (Table 1). In contrast, a robust degree of labeling was displayed throughout the dorsal lateral geniculate (dLGN) complex. Viewed at higher magnification, this dense NR2B-ir was found largely confined within many dLGN somata (Fig. 7C). This immunoreactivity was distributed fairly regularly within the entire complex (Fig. 7C,D). Note that the NR2B-ir does not fall along discrete laminae in the dLGN, in keeping with published Nissl-stained profiles of this nucleus, which do not exhibit lamination in the rat (e.g., see Fig. 2.2G–L and section 9.1.1.1 in Jones, 1985; note, however, the observations of Reese, 1988).

Ventral thalamic nuclei. Light NR2B-ir was observed in cells of the zona incerta at the rostrocaudal level of the subthalamic nucleus (Fig. 6A). The adjacent fiber systems of both the medial lemniscus (continuing from the external medullary lamina) and the cerebral peduncle were relatively understained; thus, these systems, coursing just dorsal and ventral to this region, respectively, appeared to bracket the immunopositive incertal and subthalamic cells by their reduced immunoreactivity (Fig. 6A).

Uniformly distributed, immunostained neurons in the reticular thalamic nucleus (RTN) displayed robust NR2B labeling at the rostrocaudal levels analyzed (Fig. 7A,B, Table 1). Labeling in these cells was obvious in their perikarya; cells were vaguely arranged in rows and were largely fusiform in appearance (Fig. 7B), as was previously described (e.g., see collected diencephalic studies of Ramón y Cajal, 1966; Spreafico et al., 1991). The background staining in the neuropil, however, was higher in IG experiments, although the internal capsule was unlabeled (Fig. 7A,B).

DISCUSSION

These results demonstrate that the NR2B NMDA-R subunit is present in a variety of diencephalic regions of the adult male rat. These regions include all hypothalamic and thalamic nuclei that we studied, many of which have...
Fig. 5. NR2B-immunopositive cells in epithalamic, midline, and intralaminar thalamic nuclei; IP detection using the polyclonal NR2B antibody. Coronal plane, thionin counterstain (faint gray cells). 


B: NR2B-immunoreactive cells of the thalamic paraventricular nucleus. 

C: NR2B-immunopositive cells in the reuniens (RE), rhomboid (RH), submedial (SUB), and xiphoid (XI) thalamic nuclei. 

D: Map adapted from Paxinos and Watson (1998) outlining the nuclear borders of the field shown in C. Scale bars = 100 μm in A,C; 25 μm in B.
Fig. 6. Zona incerta and selected dorsal thalamic nuclei; coronal plane (A,C,E). IP-labeled NR2B-ir cells of the zona incerta (A) and fluorescently labeled NR2B-ir cells of the parataenial (C) and anterodorsal/anteroventral thalamic (E) nuclei with use of the polyclonal NR2B antibody. The image in A was counterstained with thionin (faint gray cells). B,D,F: Maps outlining fields shown in A, C, and E, respectively. (Maps were modified from Swanson LW [1998/1999] Brain maps: structure of the rat brain, 2nd ed. Amsterdam: Elsevier [CD-ROM graphic files].) Rostrocaudal levels for each photograph on the left are accurately matched to the appropriate atlas map level on the right. B: Level 33; D: level 23; F: level 26. Scale bars = 100 µm.
Fig. 7. NR2B-ir in the reticular (A,B) and dorsal lateral geniculate (C,D) nuclei of the thalamus using the polyclonal NR2B antibody. Coronal plane. A,B: Silver-intensified gold-labeled reticular cells immunoreactive for NR2B. Note the arrangement of reticular nucleus cells along diagonally oriented rows (B). C,D: IP-labeled geniculate cells immunoreactive for NR2B, with thionin counterstain (faint gray cells). Note robust NR2B-ir within somata of geniculate cells (C). The rectangles in A and D outline the fields enlarged in B and C, respectively. Asterisk, blood vessel. Scale bars = 300 μm in A; 50 μm in B,C; 100 μm in D.
been shown here for the first time to contain the NR2B subunit. The distribution of NR2B labeling produced by the monoclonal and polyclonal antibodies was similar. The specificity of the polyclonal antibody was demonstrated by the lack of NR2B-ir observed in sections incubated either without the primary antibody or with the primary antibody preadsorbed with its fusion protein. In addition, immunoblotting studies have demonstrated the specificity of the polyclonal antibody we used (Snell et al., 1996; Currás and Dao, 1998; Currás-Collazo and Dao, 1999; Khan et al., 1999a). Furthermore, the monoclonal NR2B antibody we used reportedly does not cross-react with the NR1, NR2A, NR2C, or NR2D proteins expressed by transected cells (B. Wolfe, unpublished observations).

The earliest evidence for the existence of dienecphalic NR2B subunits came from studies examining NR2B mRNA throughout the entire brain (Kutsuwada et al., 1992; Ishii et al., 1993), but this distribution was not comprehensively resolved to the level of specific nuclei. A few reports have examined drug-induced changes in NR2B mRNA expression in whole hypothalamic tissue (Le Grevès et al., 1997, 1998), but detailed immunohistochemical reports of NR2 subunit expression/distribution within the hypothalamus have been especially lacking. With few exceptions (Watanabe et al., 1993; Cannon and Rea, 1994; Al-Ghoul et al., 1997; Herman et al., 2000), NRMDA-R subunit localization studies using intact dienecphalic tissue have been focused either on thalamic structures (Liu, 1997; Clarke and Bolam, 1998; Jones et al., 1998; Tighilet et al., 1998) and/or on subunits other than NR2B (van den Pol et al., 1994; Kus et al., 1995; Liu, 1997; Gu et al., 1999; Paquet and Smith, 2000). Petralia et al. (1994b) were the first to tabulate carefully the distribution of putative NR2B protein in rat hypothalamic and thalamic nuclei, but their antibody did not distinguish NR2A from NR2B. Our results are generally in agreement with their findings and extend them by providing strong evidence for NR2B-specific immunolabeling and for novel NR2B presence within additional dienecphalic regions. Furthermore, our data on the distribution of NR2B protein in various thalamic sites generally conform to the NR2B mRNA distribution reported in thalamic sites by Jones et al. (1998).

**NR2B expression in the hypothalamus**

Biochemical studies have implicated Glu as a transmitter released by many afferent pathways to the hypothalamus (Meeker and Myers, 1979; Walaas and Fonnum, 1980). Glu and t-aspartate, both of which can activate NRMDA-Rs (Patneau and Mayer, 1990; Currás and Dingledine, 1992), trigger excitation in hypothalamic cells (Arnauld et al., 1983) and have been described within presynaptic axons and cell bodies within several hypothalamic nuclei (van den Pol, 1990; Goldsmith et al., 1994). These nuclei include those of the magnocellular neurosecretory system, which contain a high density of Glu presynaptic terminals (Meeker et al., 1991; Decavel and van den Pol, 1992). t-aspartate, a putative neurotransmitter candidate that can also activate NRMDA-Rs, has also been found in some of these nuclei (Schell et al., 1997). Experiments employing radioligand binding (see, e.g., Meeker et al., 1994), Ca$^{2+}$ imaging (see, e.g., van den Pol et al., 1990; Dayanithi et al., 1995, Meeker et al., 1999), and electrophysiological recordings (see, e.g., Gribkoff, 1991; Hu and Bourque, 1992; Dudek et al., 1993; Ding et al., 1994; Yang et al., 1994, 1995; Yang and Hatton, 1997; Currás et al., 1998; Meeker et al., 1999; Spergel et al., 1999) all provide evidence for NMDA-R involvement in hypothalamic function.

The most robust hypothalamic expression of the NR2B subunit was observed in the ARH and in the hypothalamic magnocellular neurosecretory system, including magnocellular cells of the PVH, SON, and scattered perivascular nuclei. These latter nuclei include the NC, AFN, and LHPN. Interestingly, our demonstration of the NR2B subunit in all of these magnocellular nuclei suggests that, despite their distinct patterns of innervation (Duan and Ju, 1998; Hatton, 1990), these nuclei may each receive inputs from neurons that use Glu or other endogenous NMDA-R agonists as neurotransmitters.

PVH. Our results, demonstrating robust NR2B-ir in this nucleus, coincide with our immunoblotting evidence for NR2B protein in the PVH (Currás and Dao, 1998; Currás-Collazo and Dao, 1999) and extend the work of Petralia et al. (1994b), who reported immunoreactivity in the PVH to an NR2A/NR2B antibody. The robust presence of NR2B-ir that we observed in the PVH has been confirmed in VP- and OT-neurophysin-immunoreactive cells of this nucleus (Currás-Collazo et al., 2000). It has been recently reported that NR2B mRNA is widely expressed throughout the PVH, with the highest levels present in magnocellular cells (Herman et al., 2000). Our data, which reveal robust NR2B-ir in magnocellular PVH cells, not only confirm this finding at the protein level but reinforce the possibility that NR2B subunits are present within functional NRMDA-Rs in the PVH.

In support of this is the parallel distribution of NR1 subunit expression in the PVH (van den Pol et al., 1994; Petralia et al., 1994a; Bhat et al., 1995; Decavel and Currás, 1997; Durgam and Mifflin, 1998; Zhu et al., 1999; Paquet and Smith, 2000). NR1/NR2B subunit combinations may be sufficient to provide functional NMDA-Rs in vivo (Forrest et al., 1994; Williams et al., 1996), insofar as these subunits contain glycine and Glu binding sites, respectively (Kuryatov et al., 1994; Williams et al., 1996; Laube et al., 1997). Both of these sites must be occupied for gating of the NRMDA-R channel to occur (Kleckner and Dingledine, 1988; Currás and Pallotta, 1996).

Direct evidence for functional NRMDA-Rs in the PVH has been provided by electrophysiological data. For example, NRMDA-Rs mediate components of synaptic responses in both magnocellular and parvocellular PVH cells (Wuarin and Dudek, 1991; Boudaba et al., 1997; Daftary et al., 1998, 1999). NRMDA-R activation can also modulate GABA receptor-mediated events in some PVH cells (Bains and Ferguson, 1997). Similar modulatory events may help sculpt postsynaptic GABA$_A$ receptor-mediated effects in PVH cells that are produced by electrical stimulation of the SCH (Hermes et al., 1996). In other PVH cells, activated NRMDA-Rs trigger long-duration plateau depolarizations, leading to a large increase in action potential frequency (Bains and Ferguson, 1998), and can trigger tetrodotoxin-resistant Ca$^{2+}$ spikes, which may play a role in the dendritic release of peptides from these cells (Bains and Ferguson, 1999). Collectively, these findings highlight the diversity of events mediated by NRMDA-Rs in the PVH. Given the robust expression of NR2B in the PVH and the moderate current decay time and high Ca$^{2+}$ permeability of NR2B-containing NRMDA-Rs (Monyer et al., 1994; Grant et al., 1998), it is
NR2B-containing NMDA-Rs are involved in osmoregulation (et al., 1998; Meeker et al., 1999), support the idea that NMDA-R activation triggers VP release in the SON (et al., 1999). These results, as well as those demonstrating that NMDA-R activation triggers VP release in SON magnocellular cells (Morsette et al., 1998; Swenson et al., 1998; Meeker et al., 1999), support the idea that NR2B-containing NMDA-Rs are involved in osmoregulation. NR2B subunits may also be involved in developmental processes within the SON (Curra´s and Dao, 1998).

Insofar as our data reveal that NR2B is present in the SON dendritic zone, another possible role for NR2B involves the afferents terminating within this region. Dendrites here are likely to receive both glutamatergic input from the main and/or accessory olfactory bulb and histaminergic input from the tuberomammillary nucleus (for review see Hatton and Li, 1998). Whereas activity from olfactory bulb afferents excites NMDA-Rs of both OT and VP neurons (Yang et al., 1995), tuberomammillary-derived histamine (HA) acts differentially on the two cell types, exciting VP neurons via H1 receptors (Armstrong and Sladek, 1985; Hatton and Yang, 1996) and inhibiting OT neurons via H2 receptors and/or a HA-gated chloride channel (Yang and Hatton, 1994). Because HA enhances NMDA-R-linked activity (e.g., see Bekkers, 1993; Brown et al., 1995; Yanovsky et al., 1995, Sharonova et al., 1996), and selectively modulates NMDA-Rs containing the NR2B subunit (Williams, 1994), it is possible that HA-mediated excitation of VP cells in the SON also involves a direct action of HA on NR2B-containing NMDA-Rs. In the context of this hypothesis, any enhancement by HA of currents through NR2B-containing NMDA-Rs on OT cells (assuming that they are even activated) may not be sufficient to overcome the chloride conductance triggered separately in these cells by HA.

Finally, as discussed earlier for PVH cells, NR2B in the SON dendritic zone may be involved in mediating the dendritic release of peptides. Glu, acting via NMDA-Rs, can cause OT and VP release from dense-core vesicles within magnocellular dendrites (reviewed by Morris et al., 2000). In addition, steroids such as 17β-estradiol can exert a rapid, nongenomic action upon SON dendrites, triggering exocytosis of OT- and VP-containing vesicles that can be arrested by an NMDA-R antagonist (see Salmond et al., 1999, and Fig. 1 of Morris et al., 2000). The possibility that NR2B-containing NMDA-Rs in SON dendrites can mediate steroid-induced exocytosis is supported by evidence that pregnenolone sulfate can potentiate Ca2+ influx through recombinant NR2B-containing NMDA-Rs (Mukai et al., 2000). Although these and other data (Foy et al., 1999) demonstrate a modulatory effect of steroids on NMDA-R activity, it is not clear whether such a nongenomic effect is due to a direct action of steroids with the NMDA-R itself.

**Perivascular “accessory” magnocellular nuclei (NC, AFN, LHNP).** Except for a report mentioning immunoreactivity to an NR2A/NR2B-selective antibody within the NC (Petralia et al., 1994b), reports from our laboratories (present study; Curra´s-Collazo et al., 2000) are the first to document the specific presence of robust NR2B-ir in many perivascular magnocellular “accessory” groups characterized by Peterson (1986). In addition to the NC, we found robust NR2B-ir in the AFN, LHNP, and scattered magnocellular cells apposed to blood vessels. These cell groups have received relatively little attention in the literature, probably because of their small size and the difficulty in recording electrical activity from them. Among these nuclei, the NC is the best understood. Its neurons are tightly packed monopolar cells that respond to acute/chronic in vivo dehydration with intra- and intercellular changes visible at the ultrastructural level (Tweedle and Hatton, 1976), although it is still unknown whether the NC is a true osmoreceptor (Hatton, 1976). Also, many perivascu-
lar nuclei, including the NC, are metabolically activated during acute glucose deprivation induced by peripheral injection of the antimetabolite 2-deoxy-D-glucose (Briski and Brandt, 2000). Given the robust NR2B-ir we find in the NC, it is likely that functional NMDA-Rs are present on NC cells and that these NMDA-Rs are involved in the responses of this nucleus to osmotic and metabolic stimuli.

**SCH.** It is well-established that Glu or a similar excitatory amino acid mediates the transfer of photic information from the retinohypothalamic tract to the SCH (see Ebling, 1996, for a review) and that SCH NMDA-Rs help to receive and process this photic input (Ohi et al., 1991; Takeuchi et al., 1991; Amir, 1992, Colwell and Menaker, 1992). Some evidence suggests, however, that NMDA-R activation under these conditions is restricted to only portions of the SCH and/or to a particular circadian phase (Abe et al., 1991; Guido et al., 1999). NMDA-R activation in SCH neurons has been linked to cellular metabolism (Shibata et al., 1992), immediate-early gene expression (Abe et al., 1991; Schurov et al., 1999; but see Vuiliez et al., 1998), and Glu release (Hamada et al., 1998) and also plays a role in resetting/phase-shifting circadian rhythms already salient (Ding et al., 1994; Shibata et al., 1994; Mintz et al., 1999).

Despite the wealth of information concerning NMDA-Rs in the SCH, little is known about their subunit composition. Data concerning NR2B expression in the SCH are conflicting: Some suggest little or no NR2B mRNA in this nucleus (Mikkelsen et al., 1993, 1995; Gannon and Rea, 1994; Watanabe et al., 1993), whereas we and others find robust NR2B expression at both the mRNA and the protein levels (Petralia et al., 1994b; O'Hara et al., 1995). These differences may be due to the circadian time in which tissue was sampled, as reported for other NMDA-R subunits (Ishida et al., 1994; but see Gannon and Rea, 1994). Our data suggest that NR2B is likely a component of at least some NMDA-Rs involved in mediating the photic entrainment of SCH neurons. This is also indirectly suggested by the report that HA phase shifts the circadian pacemaker in hamsters by a mechanism that may involve direct actions at NMDA-Rs (Meyer et al., 1998). A direct action of HA at NMDA-Rs may be an indicator of NR2B presence in these cells (Williams, 1994; Sharonova et al., 1996).

**Other hypothalamic regions.** Little is known about the NR2B subunit in other hypothalamic areas. Anteriorly in the preoptic hypothalamus, where we found low to moderate NR2B levels, Adams et al. (1999) found perinatal changes in NR2B expression in neurons that are likely to influence the excitability of preoptic gonadotropin-releasing hormone neurons. Although NR2B is present in such neurons, its function in these neurons is not yet entirely clear (Gore et al., 1996; Bourguignon et al., 1997; Spergel et al., 1999).

More caudolaterally, cells of the lateral hypothalamic area (LHA) appear to be vested with NR2B-containing NMDA-Rs, some of which may mediate signals triggering eating behavior. Specifically, we have shown that intense eating behavior can be rapidly triggered in satiated rats by acute microinjection of Glu or NMDA into the LHA (Stanley et al., 1993a). This eating response appears to be largely specific to stimulation of the LHA; injections of these agents into sites surrounding this area are virtually ineffective in eliciting eating, arguing against diffusion of the agents to nearby sites (Stanley et al., 1993b). Further-
VPN. Stimulation of low-threshold somatosensory or visual inputs to VPN thalamic cells (ventral postero medial (VPM), ventral postero lateral (VPL)) has been demonstrated to evoke NMDA-R-mediated responses (reviewed by Salt and Eaton, 1996). During the first 2 weeks of postnatal development, NMDA-Rs carry the bulk of the EPSCs recorded from VPN neurons receiving cortical input, although the NMDA-R contribution to the overall current in these cells decreases after this time (Golshani et al., 1998).

In the present study, we have found that low to moderate NR2B-ir exists in the VPN. The NR1 subunit has been localized to presynaptic terminals within the VPN (Khara zia et al., 1995), and the mRNA for all NR2 subunits has been found in the VPN of primates (Jones et al., 1998). Furthermore, ultrastructure-level quantitation has revealed that 10–29% of sampled VPL synapses immunopositive for NR1 were lemniscal, whereas 60–70% were corticothalamic (Liu, 1997). Collectively, these data suggest that functional heteromeric NMDA-Rs of many subunit combinations can potentially exist in VPN cells that receive inputs from the neocortex and/or one or more sensory modalities. It is not yet clear, however, whether ascending viscerosensory information, transmitted to the neocortex by way of VPN relay cells, is driven by VPN NMDA-Rs (e.g., gustatory, gastric, or cardiopulmonary; see Cechetto and Super, 1987). NMDA-R-mediated excitation of VPN cells, perhaps involving the NR2B subunit, may not only influence cortical activity but help to drive GABAergic neurons of the RTN to influence NMDA-R-dependent spindle oscillations, as has been shown to occur between VPN and RTN cells with electrical stimulation (Warren et al., 1994).

LGN. We found relatively low levels of the NR2B subunit in the ventral LGN, and robust levels of this subunit in the dLGN, where a role for NMDA-Rs in mediating normal visual responses is well-established in several species (e.g., see Heggelund and Hartveit, 1990; Hartveit and Heggelund, 1990; Sillitto et al., 1990; Funke et al., 1991; Kwon et al., 1991). Subsets of dLGN neurons in certain species (e.g., designated “X” or “Y” cells depending on their capacity to sum spatially a visual stimulus applied to their receptive fields (see Fig. 9.21 of Jones, 1985). Visual responses evoked in both X and Y cells of the feline dLGN are markedly sensitive to NMDA-R antagonists (Scharf man et al., 1990; Sillitto et al., 1990; Kwon et al., 1991; Funke and Eysel, 1992). Also, dLGN NMDA-R activity is crucial for the developmental segregation of retinal afferents into discrete eye-specific laminae and their further refinement into on- and off-center sublaminae as well as for EPSC-enhancement in these cells (Hahn et al., 1991; Mooney et al., 1993; Ramoa and McCormick, 1994). NMDA-R activity also appears to affect dendritic development in these cells; an NMDA-R antagonist can increase dendritic branch points of LGN neurons in vivo (Rocha and Sur, 1995). Others have more narrowly relegated the contribution of activated NMDA-Rs in dLGN cells to the burst firing generated in these cells by a low-threshold Ca$^{2+}$ potential (Turner et al., 1994). These and a wealth of other studies demonstrate the importance of NMDA-Rs in mediating the visual responses of LGN neurons.

Virtually nothing is known, however, about the contribution of specific subunits to NMDA-R activity in the dLGN. Work suggesting NR2B involvement in LGN function comes from Ramoa and Prusky (1997), who found that dLGN cells of immature ferrets were more sensitive to ifenprodil, an NR2B-selective antagonist, than dLGN cells of adult ferrets. This suggested that NR2B subunits were more prevalent in the juvenile animal. If this generalizes to rats as well, these data would suggest that the NR2B-ir we find in the rat dLGN may be less robust than that observed in immature rats.

Recently, Jones and colleagues have reported that NR2B, as well as NR1, NR2A, and NR2D, is expressed at low to moderate levels in the primate dLGN and that their expression is associated with the presence of various isoforms of type II Ca$^{2+}$/calmodulin-dependent protein kinase (CaMKII). Moreover, NMDA-R subunits as well as CaMKII isoforms in the dLGN are down-regulated by visual deprivation, suggesting functional involvement of these macromolecules in the normal visual response (Tighilet et al., 1998). These findings are intriguing not only in that CaMKII isoforms are enriched at glutamatergic synapses (see Tighilet et al., 1998, for references) but because both NR2B and CaMKII are enriched in postsynaptic densities (Kennedy et al., 1983; Kelly et al., 1984; Goldenring et al., 1984; Moon et al., 1994) and are often associated together within them (Gardoni et al., 1998; Strack and Colbran, 1998) and because NR2B contains a phosphorylation site for CaMKII (Onkumar et al., 1996). The presence of CaMKII may be a useful clue to the possible presence of the NR2B subunit.

Other thalamic regions. We found moderate to fairly strong levels of NR2B-ir in the PVT and lower levels of NR2B-ir in the RE, implicating this subunit in NMDA-R-mediated functions associated with these nuclei. For example, injections of NMDA have been shown to induce kindling and seizure activity when injected into the RE (Hirayasu and Wada, 1992); NR2B may be part of the functional NMDA-Rs mediating this effect. Also, the PVT has been suggested as an important functional component of the mammalian circadian timing system and receives input from the retina (Speh and Moore, 1982), SCH (Watts et al., 1987), and intergeniculate leaflet (Weis et al., 1992). In turn, the PVT projects to a number of areas, including several hypothalamic regions (Moga et al., 1995). This may be of functional significance not only in the context of circadian timing but in the control of food intake as well, insofar as the hypothalamic targets of PVT innervation include regions such as the LHA and VMH, which are well known to be important for feeding control. We have recently demonstrated that NMDA reverse dialyzed into the LHA elicits eating that is associated with the robust expression of Fos protein in a number of diencephalic sites, including the PVT (Khan et al., 1999b). Indeed, both the PVT and the RE appear to be important in eating control and the maintenance of energy expenditure, because discrete lesions of the RE increase daily torpor, food consumption, and body weight in Siberian hamsters, effects that are prevented when the PVT is lesioned in the same animals as well (Purvis and Duncan, 1997). Lesions of the PVT in rats also produce increases in food intake and body weight (Bratnagar and Dallman, 1999).

CONCLUSIONS

Our data suggest that the NR2B subunit of the NMDA-R participates in a wide variety of diencephalic functions, ranging from sensory information processing to hormone release. More generally, if the presence of NR2B
is grossly indicative of the presence of functional NMDA-Rs, these data suggest that the contributions of NMDA-Rs to hypothalamic and thalamic cellular function are both numerous and varied. However, except for a few nuclei with which some progress has been made, our understanding of such contributions is still in its infancy. Not surprisingly, then, the goal of resolving the relative contributions of particular subunits of dienephalic NMDA-Rs still remains largely unrealized. Gaining such knowledge about these receptors is critical to our understanding of the dynamics of a neurotransmitter system that otherwise has had an elusive functional anatomy, which, simply by its ubiquity, does not readily lend itself to discrete anatomical parcellation. Improved immunohistochemical techniques (see Nuesser, 2000) are already aiding efforts to obtain the high-resolution localization of NMDA-Rs in various tissues. Also, the continuing development of NMDA-R subunit-selective antibodies and antagonists will help to unravel the contributions of particular NMDA-R subunits to the many dienephalic functions mediated by Glu and/or other EAAs as well as to certain NMDA-R-linked pathophysiological processes that can occur in dienephalic cells. For example, NMDA-R activity may underlie thalamic involvement in seizure activity or other “dysrhythmias” (Patel et al., 1988; Clifford et al., 1989; Hirayasu and Wada, 1992; Banerjee and Sneed, 1995; Koerner et al., 1996; McCormick, 1995). Additionally, NMDA-Rs may be involved in exacerbating hypothalamic susceptibility to invasion by the HIV virus (Lipton, 1992; Corasaniti et al., 1995; Lortholary et al., 1996; Raber et al., 1996; Toggas et al., 1996; Xin et al., 1999). Intriguingly, however, recombinant heteromeric NMDA-Rs containing NR2B are reportedly most sensitive to high concentrations of a synthetic peptide derived from an HIV-1 coat protein, which inhibits NMDA-R activity (Wittekindt et al., 2000). Also, NMDA-R overactivation may not be as effective at producing excitotoxic damage in certain hypothalamic neuroendocrine cells as it is in many forebrain neurons (Ebling et al., 1998; Pak and Curras, 1998).

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