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Journal of Chemical Neuroanatomy 29 (2005) 157–172
Journal of CHEMICAL NEUROANATOMY
www.elsevier.com/locate/jchemneu

Distribution of the NMDA receptor NR3A subunit in the adult pig-tail macaque brain

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Received 9 July 2004; received in revised form 24 November 2004; accepted 24 November 2004
Available online 24 December 2004

Abstract

The NMDA subtype of glutamate receptors are heteromeric complexes comprised of multiple subunits encoded by at least seven different genes (NR1, NR2A–2D and NR3A–3B), and differential expression of these subunits alters the pharmacological and electrophysiological properties of NMDA receptors. NR3A is a recently identified unique modulatory subunit that decreases NMDA receptor current and calcium influx. In rodents, NR3A is developmentally expressed, displaying robust expression early in development that declines with age, reaching low levels in the adult brain. A distinct and highly selective pattern of expression is observed in the developing and mature rodent brain, suggesting that NR3A may play a very specific role in NMDA receptor-mediated processes. NR3A expression in other species, however, is unknown. Therefore, we examined the expression of NR3A mRNA and protein in the adult macaque brain. Our results indicate that NR3A mRNA is expressed throughout much of the adult primate brain, and at high levels in specific brain regions including the neocortex, substantia nigra par compacta and cerebellum, as well as select areas of the hippocampus, amygdala, thalamus and hypothamalamus. Western blot analysis reflects that this protein is translated and expressed in multiple brain regions. In contrast to the rat mRNA, our results suggest that NR3A transcript is widely expressed in the adult primate brain. Particular enrichment in some brain areas may reflect brain-region or circuit-specific functions for this NMDA receptor subunit.

Keywords: Glutamate; Primate; Monkey

1. Introduction

Glutamate is the major excitatory neurotransmitter in the brain, and acts by stimulating both ionotropic and metabotropic glutamate receptors (Hollmann and Heinemann, 1994). Ionotropic glutamate receptors are ligand-gated ion channels that are subdivided into three classes, kainate, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and *N*-methyl-D-aspartate (NMDA), based on differing pharmacological and electrophysiological profiles. Metabotropic glutamate receptors are G-protein coupled seven-transmembrane domain receptors. NMDA receptors, in particular, have been found to play critical roles in a number of processes involved with development, synaptic plasticity, and excitotoxicity. Disturbances in NMDA receptor function are implicated in a number of neurological disorders, such as epilepsy, and in psychiatric illnesses, including schizophrenia, mood disorders, and substance abuse (Chapman, 2000; Coyle et al., 2003; Siggins et al., 2003; Zarate et al., 2003).

NMDA receptors display unique features that distinguish them from the other ionotropic glutamate receptors, and may underlie their specific functional roles (Cull-Candy et al., 2001). Unlike AMPA and kainate receptors, NMDA receptor activation requires binding of both glutamate and the co-agonist glycine, as well as the extrusion of Mg^{2+}, which blocks the channel at rest, by partial membrane depolarization. NMDA receptors are also highly permeable to Ca^{2+}, and contain a number of allosteric modulatory sites. NMDA receptors are heteromeric complexes comprised of multiple subunits encoded by at least seven different genes (NR1, NR2A–2D, and NR3A–3B) (Hollmann and Heinemann, 1994; Ciabarra et al., 1995; Sucher et al., 1995; Das

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doi:10.1016/j.jchemneu.2004.11.002

et al., 1998; Chatterton et al., 2002). Both the obligate NR1 subunit, which can be alternatively spliced to produce eight different isoforms, and the NR2 subunits can alter the activity, sensitivity and/or efficiency of NMDA receptors (Cull-Candy et al., 2001). NR1/NR2A containing receptors, for example, are more strongly blocked by Mg^{2+} compared to NR1/NR2C containing receptors, and exhibit a lower affinity for glycine compared to NR1/NR2B containing receptors (Cull-Candy et al., 2001). Due to the functional significance of this molecular diversity, subunit composition is an important mechanism for regulating NMDA receptor function.

NR3A is a modulatory NMDA receptor subunit that significantly alters these features when incorporated into a functional receptor. Co-assembly of NR3A with NR1 and NR2 subunits decreases receptor current, Ca^{2+} influx, and Mg^{2+} sensitivity, and increases the mean open time of the channel (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998). NR3A interacts directly with protein phosphatase 2A (PP2A), and can dephosphorylate NR1 at serine 897 (Chan and Sucher, 2001). Since protein phosphorylation is thought to be an important regulatory mechanism, NR3A may regulate NMDA receptor activity by decreasing the phosphorylation state of the NR1 subunit.

Of particular interest is the finding that NR3A can co-assemble with the NR1 subunit to form a non-conventional NMDA receptor that acts as an excitatory glycine receptor (Chatterton et al., 2002). This NR3/NR1-containing NMDA receptor binds glycine, but not glutamate, and glycine alone is sufficient to elicit a physiologically relevant current that is impermeable to Ca^{2+}. While the functional role of these receptors remains to be determined, ion channels displaying these electrophysiological characteristics have been identified in rat cortical cultures, suggesting that NR3/NR1-containing NMDA receptors are present in vivo (Chatterton et al., 2002).

In rodents, NR3A is a developmentally regulated subunit, that has been reported to be particularly enriched in regions such as the neocortex, thalamus, amygdala, and subiculum (Ciabarra et al., 1995; Sucher et al., 1995; Wong et al., 2002). NR3A is robustly expressed early in brain development, peaking around postnatal day 7, and subsequently declines with age, reaching very low levels of expression in the adult rodent brain. This suggests that NR3A may play a key role in developmental processes mediated by NMDA receptors. In mice lacking the NR3A subunit, cortical spine density is increased, suggesting that NR3A may specifically play a role in processes associated with synapse formation including dendritic growth (Das et al., 1998).

While the human NR3A subunit has been cloned (Eriksson et al., 2002), the expression of NR3A in species other than rodents has not been examined. Consequently, this study was designed to examine the expression of NR3A in the adult pig-tail macaque brain. Serving as a marker for functional NMDA receptors, the expression of the obligate NR1 subunit was also examined and compared to that of NR3A. Given the unique properties associated with the NR3A subunit, a detailed description of its relative distribution may provide insight into understanding its functional role in glutamatergic neurotransmission, as well as possible roles in brain disorders involving NMDA receptor dysfunction.

2. Materials and methods

2.1. Tissue

Brains from adult pig-tail macaques (*Macaca nemestrina*) ranging in age from 7 to 13 years were obtained from the Regional Primate Research Center of the University of Washington. The animals had been sacrificed as part of protocols unrelated to the present study, which did not require brain tissue. Protocols involving these animals were reviewed by the Washington Primate Research Center Research Review Committee and by the University of Washington IACUC.

For in situ hybridization studies, the brains from four adult female pig-tail macaques were used. Each brain was blocked in the coronal plane into five 1.5 cm slabs comprising the entire extent of the brain from the frontal pole through the cerebellum and occipital cortex and stored at −80 °C. The blocks were cryostat sectioned (10 μm) and mounted onto poly-L-lysine subbed microscope slides, desiccated and returned to storage at −80 °C until use in hybridization studies.

2.2. In situ hybridization

Riboprobes were synthesized from linearized plasmids containing subclones for the NR1 and NR3A subunits. An NR3A subclone based on the human gene [NCBI GenBank Accession number AJ416950, nucleotide coding region (2104–2689)] was amplified by PCR from an unamplified human fetal brain library, inserted into pCR4Blunt-TOPO vector (Zero Blunt TOPO PCR cloning kit, Invitrogen, Carlsbad, CA) and the final product confirmed by sequencing. An NR1 riboprobe was synthesized from linearized plasmid in pBluescript SK containing an NR1 subclone based on the rat sequence (nucleotide-coding region 923–1383) that recognizes all eight NR1 splice variants (Ibrahim et al., 2000). Riboprobes were labeled by mixing 100 μCi of [^{35}S] UTP or [^{33}P] UTP, 2.0 μl 5× transcription buffer, 1.0 μl 0.1 M dithiothreitol (DTT), 1.0 μl each of 10 mM ATP, CTP, GTP, 2.0 μl linearized plasmid, 0.5 μl RNase inhibitor and 1.5 μl Sp6 or T7 RNA polymerase, and incubating the reaction mixture for 2 h at 37 °C. 1 μl DNase (RNase-free) was then added and incubated for an additional 15 min at room temperature. Labeled probe was then purified with a Micro Bio-Spin P-30 Tris Spin Column (Bio-Rad Laboratories) and DTT was added to a final concentration of 0.01M.

Slides were removed from −80 °C storage and fixed in 4% formaldehyde for 1 h at room temperature, followed by three rinses in 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.2). Next, they were acetylated (0.1 M triethanolamine pH 8.0: acetic anhydride (400:1, v:v) for 10 min, washed in 2× SSC for 10 min, and dehydrated in graded alcohols. 5×10^6 cpm of radiolabeled probe in 500 μl of hybridization buffer (50% formamide, 10% dextran sulfate, 3× SSC, 50 mM Na_2HPO_4, 1× Denhardt's solution, 100 μg/ml yeast tRNA, 10 mM DTT) was placed on each slide, covered and incubated overnight at 55 °C. The following day, cover slips were removed and slides were washed in 2× SSC for 5 min at room temperature followed by RNase treatment (200 μg/ml in 10 mM Tris–HCl pH 8.0, 0.5M NaCl) at 37 °C for 30 min. Slides were then washed twice in 2× SSC for 15 min, once in 1× SSC for 15 min at room temperature, followed by two consecutive washes in 0.5× SSC for 1 h each at 55 °C, and a final wash in 0.5× SSC at room temperature for 15 min. Slides were then dehydrated in graded alcohols, and apposed to X-ray film (Kodak Biomax MR-1, New England, Nuclear, Boston, MA) for 1 week (NR1) or 2 months (NR3A). As a control, additional slides were probed with an NR3A sense riboprobe.

Images were acquired by digitizing film images using a CCD camera. Structures were identified by comparing images to plates and diagrams from a standard atlas of the macaque brain (Martin and Bowden, 1996). Using Scion Image software, optical density measurements relative to background were obtained for regions of interest in each brain, and then averaged across animals for each region. Mean optical density values in each region were divided into quartiles, and a rating of relative expression was assigned to each region on a scale from 1 (+, lowest) to 4 (++++, highest) by quartile, as we have previously described (Oakman and Meador-Woodruff, 2004).

Selected hybridized slides were dipped in Kodak NTB-2 emulsion diluted 1:1 with sterile water and warmed to 40 °C. Dipped slides were stored in darkness at 4 °C and developed at weekly intervals to determine optimal exposure time. After 1 month, all slides were developed by immersion in Kodak D19 developer for 2 min, rinsed, fixed in Kodak Rapid Fix for 3 min, and washed in running deionized water. The developed slides were then counterstained by Nissl stain followed by decolorization in a series of ethanol and xylene washes. Slides were coverslipped with Permount and viewed with a Zeiss microscope. Digital images were acquired using a CCD camera.

2.3. Western blot analysis

A monoclonal NR3A antibody was used for examining NR3A protein expression (kind gift of Dr. Nikolaus Sucher, Hong Kong University of Science and Technology). Brain regions from additional female adult pig-tail macaques were dissected. Samples were weighed and homogenized in 9:1 volume of cold homogenization buffer [50 mM Tris–acetate, 10% sucrose, pH 7.4 containing 1 tablet per ml of complete protease inhibitor cocktail tablets (Roche)]. Homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants were collected and further centrifuged at 100,000 × *g* for 30 min at 4 °C. Pellets were then washed 3× in cold homogenization buffer without sucrose, and resuspended in solubilization buffer with 2% SDS [137 mM sodium chloride, 2.7 mM potassium chloride, 2.85 mM dihydrate disodium hydrogen phosphate, 1.4 mM dipotassium hydrogen phosphate, 5 mM EDTA, 5 mM EGTA, 1 mM sodium vanadate containing protease inhibitor cocktail tablets (1 tablet/ml)] and rocked overnight at 4 °C. Samples were then aliquoted and stored at −80 °C.

Total protein concentrations were determined using the BCA protein assay (Pierce). Samples were prepared by boiling 25 μg of total protein for 10 min in 2× loading buffer [126 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 1 M urea, 300 mM dithiothreitol, and 0.005% bromophenol blue]. Samples were then loaded and run on an 8% SDS-polyacrylamide gel. Proteins were then transferred to a PVDF membrane (Pierce) and blocked overnight in 5% non-fat dry milk in PBS-Tween (0.1% PBS-T) at 4 °C. The membrane was then immunoblotted by incubation with a monoclonal NR3A antibody at 1:1000 dilution in 5% non-fat dry milk in PBS-Tween for 2 h at room temperature. The blot was then rinsed 3× with PBS-Tween, washed for an additional hour in PBS-Tween, and incubated with a mouse secondary antibody (1:2000) for 1 h. The blot was then rinsed 3× with PBS-Tween, washed for 30 min in PBS-Tween and visualized using enhanced chemoluminescence (ECL) and Hyperfilm ECL (Amersham). To control for total protein loading, the blot was re-probed with a monoclonal antibody for beta tubulin (1 μg/μl, upstate).

2.4. Northern blot analysis

Brain regions from additional female adult pig-tail macaques were dissected. The brain samples were weighed and homogenized in 1 ml trizol per 100 mg of tissue. Following centrifugation at 10,000 rpm for 15 min, the aqueous phase was mixed with chloroform and re-centrifuged. RNA was precipitated with isopropanol, and the resulting pellet was washed in 75% ethanol, re-centrifuged at 5000 rpm and resuspended in 200 μl formamide. Ten micrograms of extracted total RNA was loaded and run on an agarose-formaldehyde gel and transferred overnight to hybond membrane. The membrane was then cross-linked by baking 2 h at 80 °C, pre-hybridized 2–4 h at 65 °C in a 50% formamide hybridization buffer, and incubated overnight at 65 °C with [^{33}P]-labeled NR3A riboprobe. The following day, the membrane was washed in 2× SSC for 1 h, followed by a 1 h wash in 2× SSC with 0.1% SDS and a 2 h wash at 65 °C in 0.1× SSC with 0.5% SDS, and a final wash in 2× SSC at room temperature for 30 min. The blot was then dried, and apposed to photographic film for 2 weeks.

3. Results

We confirmed the specificity of the NR3A riboprobe by examining adjacent sections incubated with either sense or antisense probes. Specific labeling was detected only in sections incubated with the antisense probe (Fig. 1, top). No significant labeling was found in adjacent sections incubated with a sense probe (Fig. 1, middle). This was further confirmed by Northern blot analysis, which identified a single mRNA species at the predicted molecular weight of 8 kb (Fig. 1, bottom). The NR1 riboprobe has previously been characterized and found to specifically label (Ibrahim et al., 2000).

The transcript for the NR3A subunit was detected in the adult pig-tail macaque brain, and its relative distribution was examined and compared to NR1 mRNA expression.

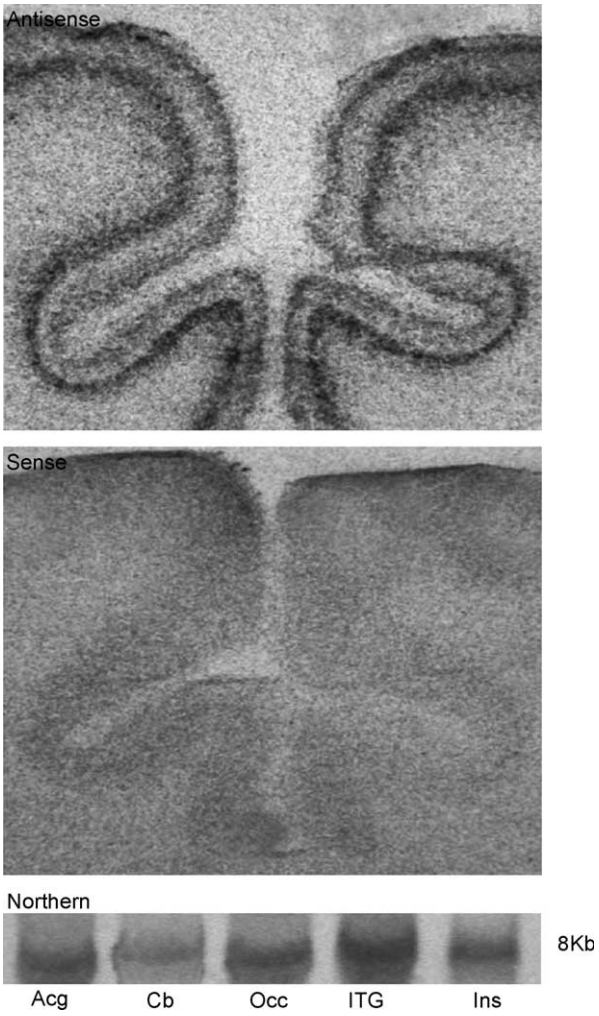

Fig. 1. Specificity of NR3A riboprobe. Specificity of the NR3A riboprobe was determined by in situ hybridization and northern blot analysis. NR3A mRNA expression was examined using [^{33}P]-labeled NR3A antisense (top panel) and sense (middle panel) riboprobes in the adult pig-tail macaque brain at the level of the anterior cingulate cortex. Specific labeling was seen only with antisense probe. Northern blot analysis using a radiolabeled NR3A antisense riboprobe identified a single mRNA species at 8 kb, consistent with the predicted size of this transcript (bottom panel).

Consistent with previous reports describing the distribution of NR1 in the rodent and primate brain, NR1 mRNA was found to be expressed at high levels throughout much of the pig-tail macaque brain (Fig. 2, left). In contrast, NR3A mRNA was heterogeneously expressed at significantly lower levels relative to NR1, although in select brain regions, prominent labeling was observed (Fig. 2, right). The expression pattern of NR3A did, however, typically coincide with NR1, consistent with NR3A being an NMDA receptor subunit. A summary of the relative regional expression of NR3A is given in the Table 1.

3.1. Forebrain

3.1.1. Neocortex

In general, NR3A is highly expressed throughout most of the cerebral neocortex, displaying a distinct laminar distribution. NR3A mRNA levels were not homogeneous across all six cortical lamina. Rather, NR3A was concentrated in layer V, creating a prominent isodense band. While less dense, a second more superficial isodense band was visible, corresponding to layer II (Fig. 3, left panel). NR3A mRNA expression in the other cortical layers was considerably less. In emulsion dipped sections, discrete grain clusters were observed over both large as well as small neurons (Fig. 3, right panel), suggesting that NR3A mRNA is expressed by both glutamatergic and GABAergic neurons in the neocortex, consistent with the cellular expression reported in the developing rodent brain. This expression pattern was present throughout most of the cerebral neocortex, including the anterior cingulate, frontal, parietal, occipital, temporal, and insular cortices. The level of expression, however, decreased in more caudal areas of the cortex, especially in the occipital lobe. In contrast, NR1 mRNA was present at high levels throughout the neocortex in most cortical laminae.

3.1.2. Basal ganglia and rostral subcortical structures

Minimal NR3A mRNA expression was found in the caudate, putamen, and globus pallidus, and in limbic-associated areas including the nucleus accumbens and substantia inominata. This was strikingly different from NR1 expression, which was detected at high levels in all of these regions (Fig. 2). Slightly higher levels of expression were detected in other limbic-associated subcortical structures, such as the medial and lateral septal nuclei and the nucleus of the stria terminalis. NR3A expression, however, was high in the claustrum.

3.1.3. Amygdala and hippocampal formation

In the amygdala, NR3A mRNA was expressed at low levels in most nuclei, with the exception of the accessory basal nucleus and the central nucleus, where it was prominently expressed (Figs. 2 and 4, top, left panel). On the other hand, NR1 mRNA was abundantly expressed in all nuclei of the amygdala (Figs. 2 and 4 top, right panel). In the

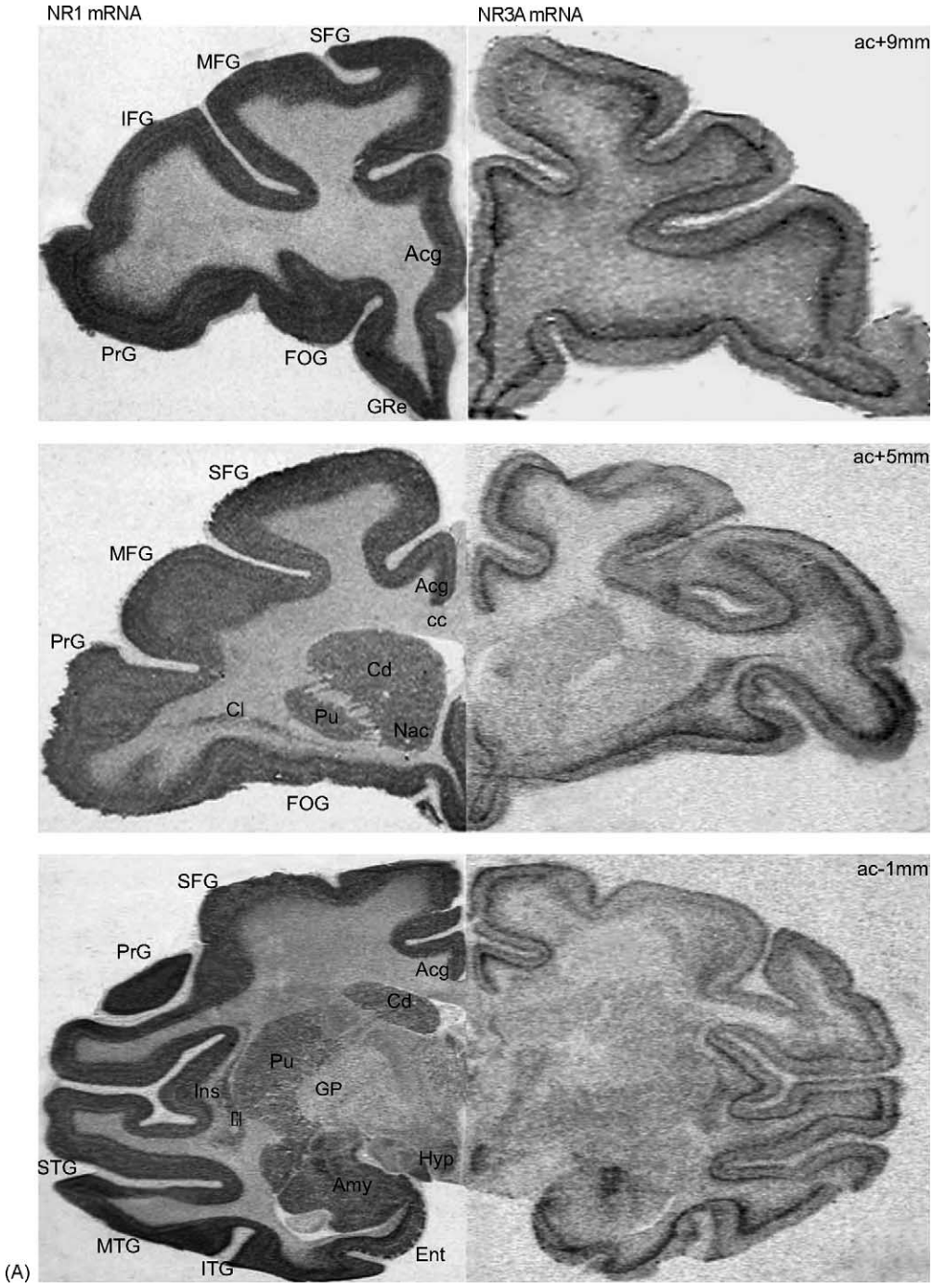

Fig. 2. Distribution of NR3A and NR1 mRNA in the adult pig-tail macaque brain. In situ hybridization was performed on coronal sections of brains from four adult female pig-tail macaques (*Macaca nemestrina*) to examine the expression of NR3A (right) and NR1 (left) transcripts throughout the extent of the brain. NR3A mRNA was expressed at low levels throughout most of the brain, although moderate levels were found in select regions. NR1, on the other hand, was expressed at high levels in many brain regions, consistent with its role as the obligate NMDA receptor subunit. The distribution of NR3A coincided with NR1, suggesting it is expressed in regions containing functional NMDA receptors. Sections are labeled as distance in millimeters rostral (+) or caudal (−) to the anterior commisure.

hippocampus, NR3A mRNA expression was low and just above background, with the exception of the CA2 subfield, which was intensely labeled (Figs. 2 and 4 bottom, left panel). Both the dentate gyrus and the subiculum were also labeled, but to a lesser degree. Similar to expression in the amygdala, NR1 mRNA was found at high levels in all regions of the hippocampal formation (Figs. 2 and 4 bottom, right panel).

3.1.4. Thalamus and hypothalamus

Expression of NR3A mRNA was low in most thalamic nuclei, although differential labeling was visible. Higher

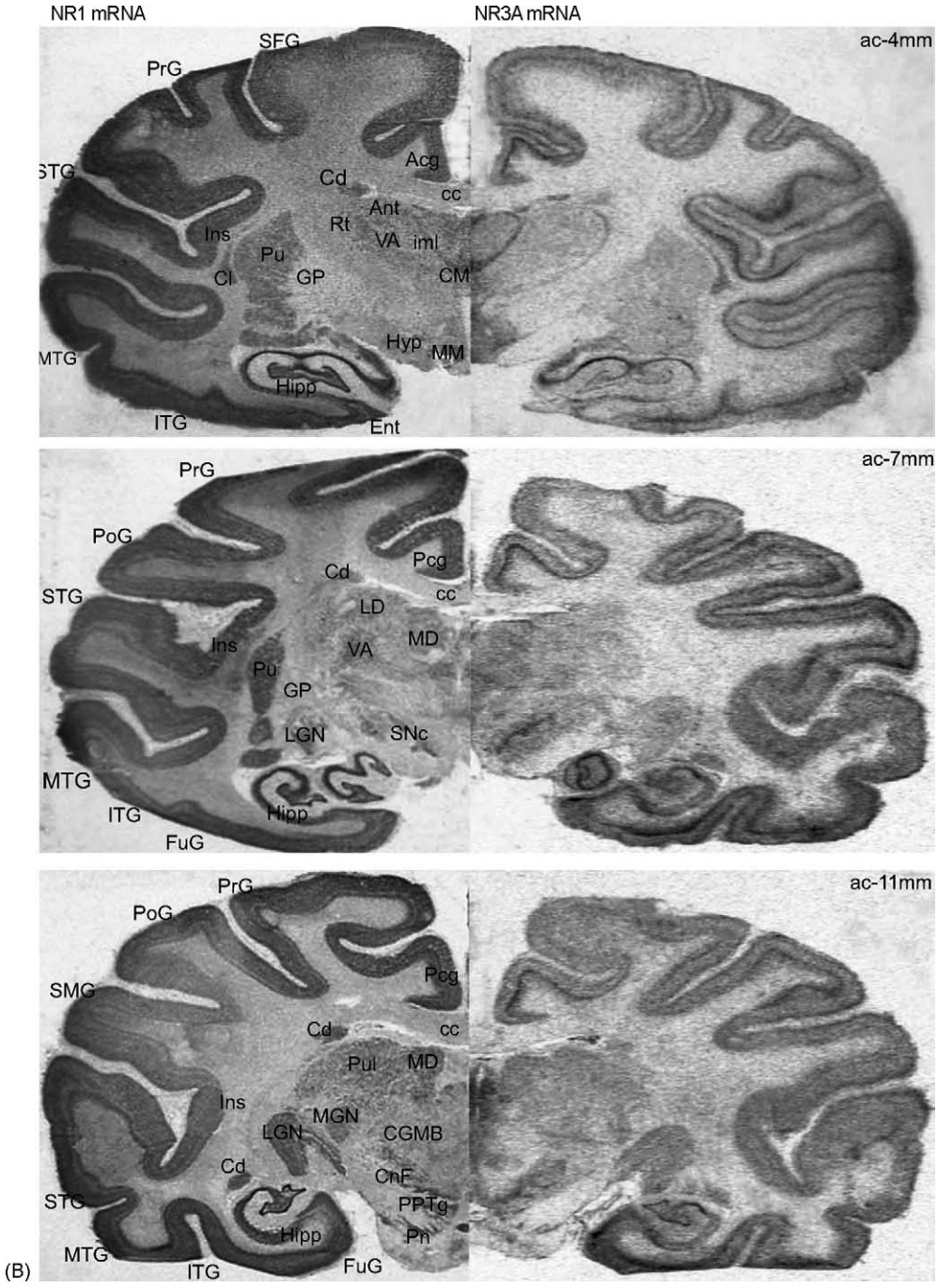

Fig. 2. (*Continued*).

levels of NR3A mRNA were observed in midline nuclei. Both the central medial and paraventricular nuclei were prominently labeled. Other medial nuclei such as the reuniens and mediodorsal nuclei were moderately labeled. Relatively high levels of NR3A mRNA were present in the reticular and intralaminar nuclei, as well as in the lateral and medial geniculate nuclei, and the pulvinar (Figs. 2 and 5). Similar to thalamic labeling, NR3A mRNA expression in the hypothalamus was enriched in midline nuclei. Notably, dense labeling was observed in the ventral medial and dorsal medial nuclei, as well as in the posterior hypothalamus and mammillary bodies (Figs. 2 and 5). Robust expression was also evident in the medial habenula of the epithalamus (Fig. 5). The subthalamic nucleus was moderately labeled (Fig. 5). The distribution of NR1 mRNA in the thalamus and hypothalamus was similar to NR3A. NR1 mRNA was present at relatively low levels compared to cortical expression, although expression was higher in comparison to NR3A mRNA (Fig. 2).

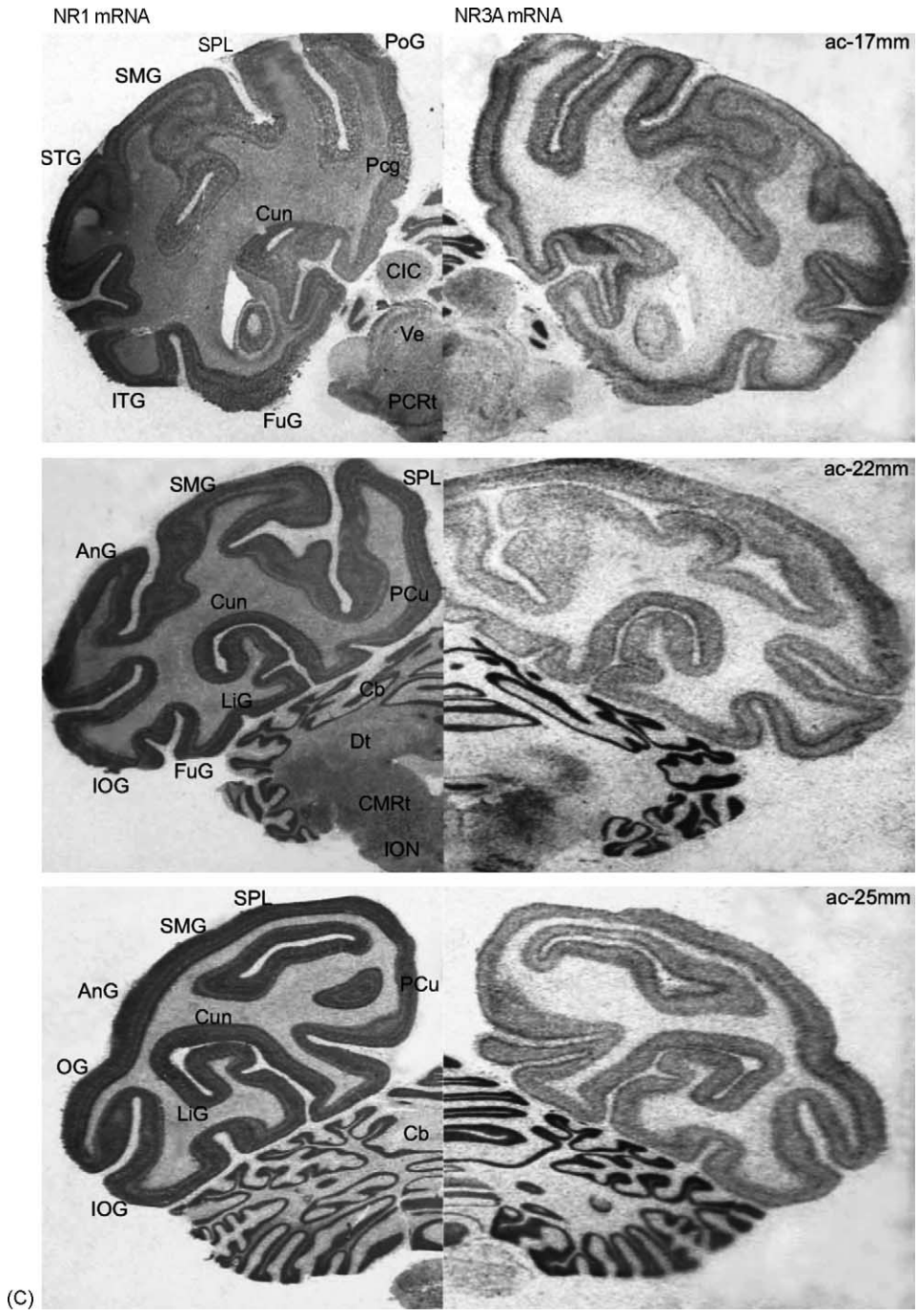

Fig. 2. (*Continued*).

3.2. Midbrain

In the midbrain, prominent NR3A mRNA expression was detected in the substantia nigra pars compacta, as well as in the red nucleus (Figs. 2 and 5). Moderate NR3A mRNA levels were detected in the central grey substance, reticular formation and the dorsal raphe nucleus. Lower NR3A mRNA levels were seen in the ventral tegmental area (Figs. 2 and 5). The distribution of NR3A mRNA in these brain regions paralleled NR1, and while NR1 mRNA was not robustly expressed in these regions relative to cortical expression, it was higher compared to NR3A mRNA (Fig. 2).

3.3. Hindbrain

At the level of the pons and medulla, NR3A mRNA was found in many structures, including the vestibular nuclei, the

Table 1
NR3A mRNA expression in the adult pig-tail macaque brain

	NR3A
Forebrain	
Cerebral cortex	
Neocortex	
Frontal lobe	+++
Parietal lobe	+++
Insula	+++
Temporal lobe	+++
Occipital lobe	+++
Cingulate gyrus	+++
Paleocortex	
Parahipppocampal gyrus	++
Archicortex	
Dentate gyrus	++
Hippocampus	
CA1	++
CA2	++++
CA3	++
CA4	++
Subiculum	+++
Basal ganglia	
Striatum	+
Nucleus accumbens	+
Globus pallidus	+
Substantia innominata	+
Amygdala	
Medial amygdaloid nucleus	
Central amygdaloid nucleus	
Accessory basal amygdaloid nucleus	
Lateral amygdaloid nucleus	
Claustrum	++
Septum	+
Thalamus	
Anterior nuclei	+
Ventral nuclei	+
Reticular formation	+
Intralaminar nuclear group	++
Paraventricular nucleus	+++
Central medial nucleus	+++
Reuniens nucleus	++
Medial dorsal nucleus	++
Lateral dorsal nucleus	+
Centromedian nucleus	+
Pulvinar	+
Lateral geniculate body	+
Medial geniculate body	+
Hypothalamus	
Anterior hypothalamic area	+
Dorsal hypothalamic area	++
Lateral hypothalamic area	++
Paraventricular nucleus	++
Dorsomedial nucleus	++++
Ventromedial nucleus	++++
Arcuate nucleus	+
Mammillary nucleus	++
Posterior hypothalamic region	+
Subthalamus	
Subthalamic nucleus	++
Epithalamus	
Habenula	+++
Pretectal region	+
Superior colliculus	+
Inferior colliculus	+
Sagulum	+
Midbrain	
Midbrain tegmentum	
Occulomotor nuclear complex	+
Midbrain reticular formation	++
Red nucleus	++
Central grey substance of the midbrain	++
Dorsal raphe nucleus	++
Substantia nigra, pars compacta	+++
Substantia nigra, pars reticulta	+
Ventral tegmental area	++
Hindbrain	
Pons	
Trigeminal nuclei	+
Pontine reticular formation	++
Superior olivary complex	+++
Locus ceruleus	+
Nucleus subcerelueus	+
Nucleus abducens	+
Pontine raphe nucleus	++
Dorsal nucleus of the lateral lemniscus	+
Trapezoid body	+
Pontine nuclei	++
Medulla	
Inferior olive	+++
Vestibular nuclei	++
Cochlear nuclei	+
Medullary reticular formation	++
Cerebellum	
Cerebellar cortex	++++
Deep cerebellar nuclei	++

superior and inferior colliculi, the pontine and inferior olivary complexes (Figs. 2 and 5). Throughout the cerebellar cortex, NR3A mRNA expression was relatively dense, and deep cerebellar nuclei expressed moderate levels of NR3A mRNA (Figs. 2 and 5).

3.4. NR3A protein expression

To verify that NR3A transcript is translated into protein, we performed Western blot analysis and identified a single band of approximately 125 kDa, consistent with previous reports of NR3A protein expression in the rodent (Fig. 6). NR3A protein expression was observed in a number of brain regions, including the prefrontal, occipital, parietal, and temporal cortices, as well as in the thalamus, hippocampus and putamen. Consistent with our transcript data, NR3A protein levels varied between brain regions, suggesting that NR3A is differentially expressed in the macaque brain.

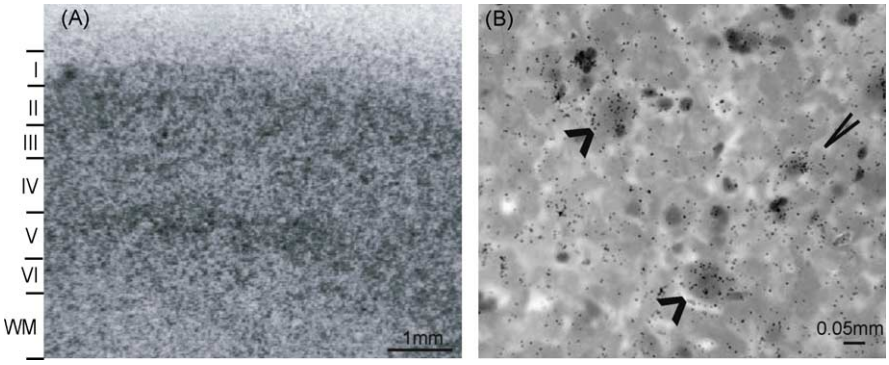

Fig. 3. Neocortical expression of NR3A. (A) NR3A mRNA expression in the neocortex showing laminar distribution. NR3A mRNA is expressed throughout the neocortex, but is most abundant in layer V. (B) High power view of the cellular distribution of NR3A mRNA in cortical layer V. NR3A mRNA is expressed in both large glutamatergic (bold arrows) and small GABAergic (fine arrow) neurons.

4. Discussion

While NR3A has previously been thought of as a developmentally regulated NMDA receptor subunit, our study indicates that NR3A expression persists into adulthood throughout the primate brain. Prominent labeling was observed across much of the neocortex, and in specific nuclei or subregions of the thalamus, hypothalamus, hippocampal formation, amygdala, midbrain, and cerebellum. This suggests that NR3A functions in a number of brain processes associated with limbic, motor, and sensory systems in the mature brain, yet may have a specific role

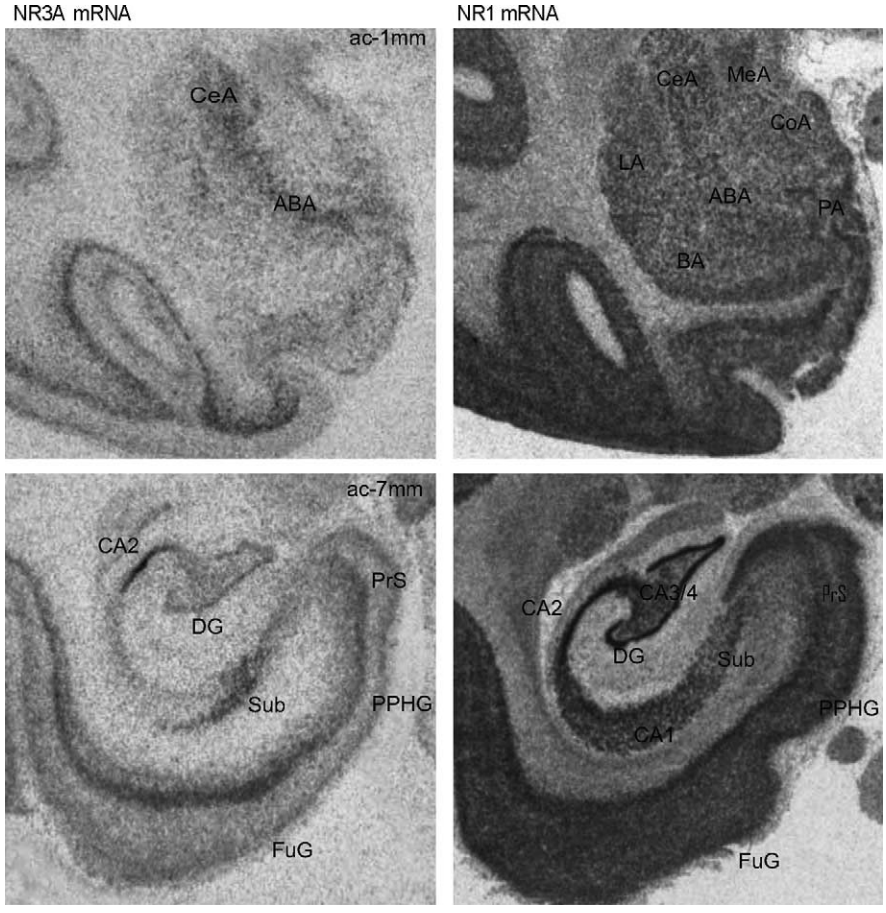

Fig. 4. NR3A and NR1 mRNA expression in the amygdala and hippocampus. NR3A mRNA expression was visualized in the amygdala (top panels) and hippocampus (bottom panels) in coronal sections from adult pig-tail macaque brains. NR3A mRNA was expressed at relatively high levels in the accessory basal nucleus (ABA) and central nucleus (CeA) of the amygdala and in the CA2 subregion of the hippocampus. NR1 mRNA was prominently expressed in all nuclei of the amygdala and subregions of the hippocampus, as well as the dentate gyrus and subiculum.

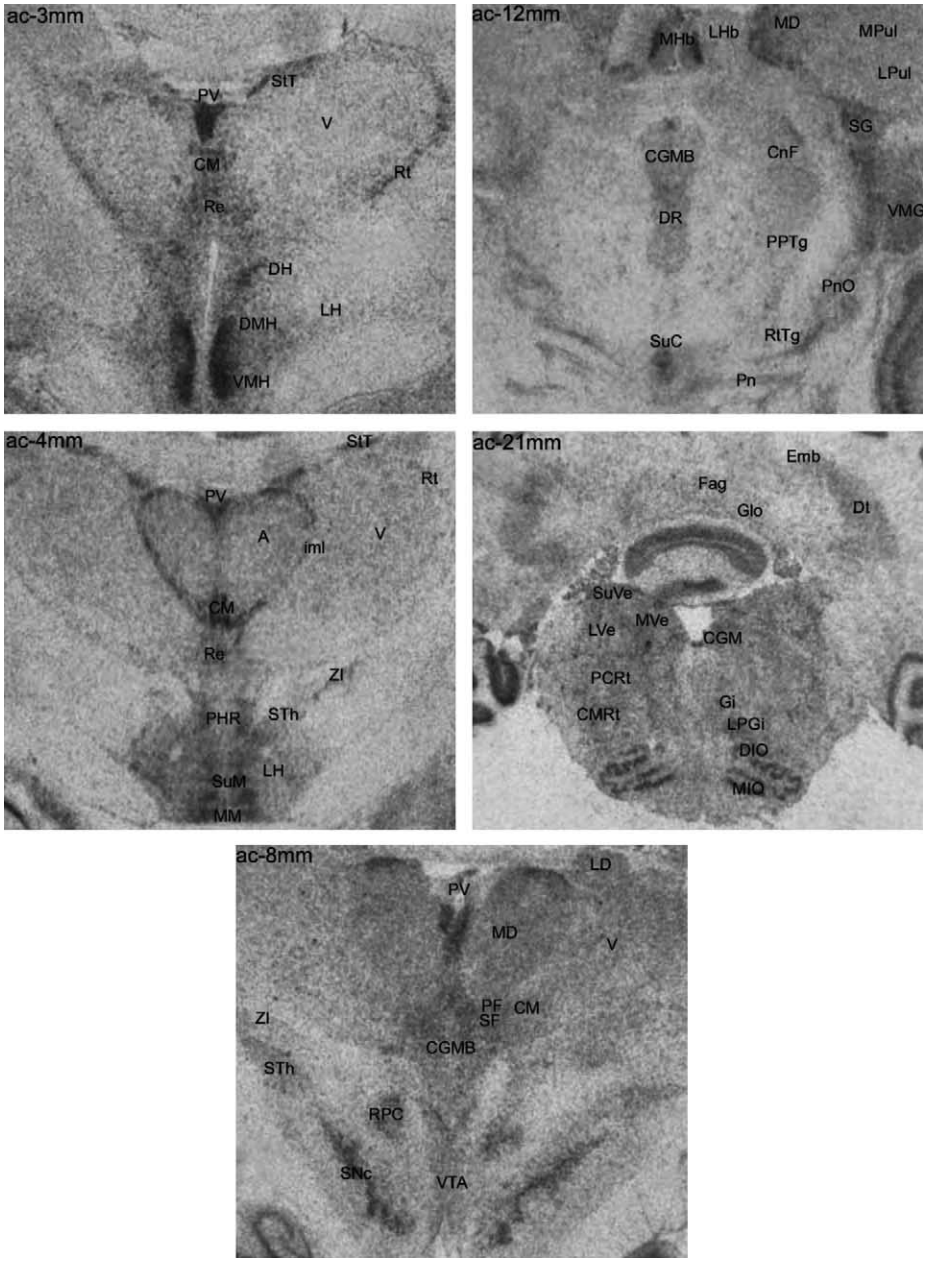

Fig. 5. Anatomical distribution of NR3A mRNA in regions of the diencephalon, midbrain, and hindbrain structures. NR3A mRNA was differentially expressed in the thalamus, hypothalamus, midbrain, and cerebellum. In the thalamus, the paraventricular, central medial, reticular, intralaminar, reuniens, medial dorsal, and pulvinar nuclei were labeled. In the hypothalamus, the dorsomedial, ventral medial, dorsal, and posterior nuclei were readily visible, as well as the mammillary bodies and the lateral hypothalamus. In the epithalamus, the medial habenula was prominently labeled. In the midbrain, several structures, including the substantia nigra pars compacta, red nucleus, central grey substance, dorsal raphe, and pontine nuclei were labeled. At the level of the cerebellum, several nuclei were labeled, most prominently the olivary complex. Sections are labeled as distance in millimeters rostral (+) or caudal (−) to the anterior commisure.

within these systems. The ability of the NR3A subunit to decrease the activity of NMDA receptors and calcium current (Ciabarra et al., 1995; Sucher et al., 1995), and possibly form non-conventional excitatory glycine receptors (Chatterton et al., 2002), suggests that this restrictive expression pattern may be important for modulating NMDA-receptor mediated processes and for neural transmission in these regions of persistent expression in the adult brain.

4.1. Neocortex

Similar to reports in the rodent brain (Ciabarra et al., 1995; Sucher et al., 1995), we found that NR3A was robustly expressed in the neocortex of the primate brain, displaying a unique laminar distribution. NR3A mRNA was enriched in layer V, with robust expression in large neurons, most likely pyramidal cells, suggesting that NR3A may regulate the

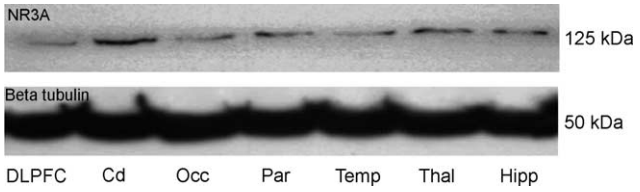

Fig. 6. NR3A protein expression. Western blot analysis was performed to examine NR3A protein expression in an adult pig-tail macaque brain. As a control, the blot was also probed for beta-tubulin. A single band at 125 kDa was identified in all brain regions examined, consistent with the predicted size of NR3A protein. *Abbreviations:* Cd, caudate; DLPFC, dosolateral prefrontal cortex; Hipp, hippocampus; Occ, occipital cortex; Par, parietal cortex; Temp, temporal cortex; Thal, thalamus.

activity of these neurons, and thus modulate a number of cortical-subcortical pathways.

Expression in layer V pyramidal neurons likely explains the mismatch we noted between transcript and protein expression. Western blot analysis indicated NR3A transcript was translated into protein in the neocortex, as well as in other brain regions, and likely can be incorporated into functional NMDA receptors. However, in contrast to our transcript data showing low levels of NR3A mRNA in the thalamus and the striatum, we observed intense protein expression in these regions, suggesting high levels of NR3A protein in the thalamus and striatum. This suggests that the transcripts for NR3A in these regions are located in cell bodies in other regions and the protein is transported to these subcortical regions. The most likely source of striatal NR3A protein is the pyramidal cells in layer V of the frontal cortex.

Multiple frontal-subcortical circuits have been identified that link the cortex to subcortical structures, specifically involving the striatum, globus pallidus, and thalamus (Tekin and Cummings, 2002). A motor circuit, originating from neurons in the supplementary motor area, premotor and somatosensory cortex, and the occulomotor circuit, originating from neurons in the frontal eye fields and posterior parietal cortex are involved in motor functions. The dorsolateral prefrontal circuit, the lateral orbital frontal circuit, and the anterior cingulate circuit serve executive functions, social behavior, and motivated states in humans. Accumulating data suggest that dysfunctions of some of these circuits may underlie psychiatric disorders, including obsessive compulsive disorder, major depression, bipolar disorder, posttraumatic stress disorder, and schizophrenia (Tekin and Cummings, 2002; Baxter, 1994; Mayberg, 1994; Goldman-Rakic and Selemon, 1997; Tamminga et al., 1992). There is also growing evidence that glutamatergic abnormalities may play a role in several of these disorders, and that this may involve alterations in glutamate receptor subunit expression (McCullumsmith et al., 2004; Paul and Skolnick, 2003; Carlsson, 2000). NR3A abnormalities may therefore play a significant role in the pathophysiology of these illnesses. In support of this, we have recently found alterations in NR3A mRNA expression in the dorsolateral prefrontal cortex in both schizophrenia and bipolar disorder relative to a comparison group (Mueller and Meador-Woodruff, 2004).

4.2. Midbrain

In the midbrain, the substantia nigra, pars compacta is the major site of dopaminergic neurons in the brain, and there is growing interest in glutamatergic regulation of midbrain dopaminergic neurotransmission, in both the axon terminals in the striatum and the cell bodies of the pars compacta (Morari et al., 1998; Naito and Kita, 1994; Sato, 1986). The prefrontal cortex, along with the subthalamic nucleus and pedunculopontine tegmental nucleus, provide glutamatergic input to dopaminergic neurons in the substantia nigra (Morari et al., 1998). The prefrontal cortex also provides glutamatergic input to the striatum (Morari et al., 1998). This suggests that NR3A may participate in the modulation of dopaminergic neurotransmission by regulating cortical activity and glutamate release. Glutamate receptors have been shown to be expressed on these nigral neurons (Counihan et al., 1998), and there are accumulating data that dopaminergic activity is mediated, in part, by NMDA receptors expressed on these neurons (Morari et al., 1998). High levels of NR3A mRNA in the pars compacta suggests that dopaminergic neurons express the NR3A subunit, and may thus directly influence midbrain dopaminergic neurotransmission.

Nigral expression of NR3A may serve a neuroprotective role. In Parkinson's disease, the dopaminergic neurons in the pars compacta degenerate (Brain 1991;114:2283–301, Brain 1999;122:1437–48), and there is increasing evidence for involvement of the glutamatergic system in the pathogenesis of this disorder (Lange et al., 1997; Greenamyre, 1993; Carlsson and Carlsson, 1990). 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) has been identified as a neurotoxin selective for melanin-containing dopaminergic neurons in humans and non-human primates, and provides a useful model for studying the pathophysiology of Parkinson's disease (Przedborski and Vila, 2003). It has been shown that MPTP-induced degeneration of dopaminergic neurons involves glutamate-mediated toxicity, destroying specifically the dopaminergic neurons in the substantia nigra (Loschmann et al., 1994). Further, NMDA antagonists can protect nigral neurons from MPTP-induced toxicity, whereas their striatal terminals still degenerate (Loschmann et al., 1994). The NMDA receptor antagonist MK-801 has been found to prevent dopamine cell loss in culture, while NMDA receptor agonists potentiate cell loss (Sonsalla et al., 1998). Given that NR3A decreases NMDA receptor activity, similar to the actions of NMDA receptor antagonists, the expression of NR3A in the neurons of the pars compacta may provide an endogenous mechanism for protecting dopaminergic neurons from glutamate excitotoxicity, and NR3A may provide a useful target for treatment of Parkinson's disease.

4.3. Dienchephalon

4.3.1. Thalamus

The thalamic midline and intralaminar nuclei have long been thought to be part of an arousing system in the brain, and are involved in cognitive, sensory and motor functions (Van der Werf et al., 2002). Individual midline and intralaminar nuclei receive distinct afferents, project to specific parts of the cerebral cortex and striatum, and participate in the functional integration of limbic cortical and striatal circuitry (Groenewegen and Berendse, 1994; Otake and Nakamura, 1998). NR3A mRNA was most abundant in these nuclei, with the strongest labeling found in the paraventricular nucleus, followed by the central medial and reuniens nuclei, suggesting that by modulating the activity of the neurons in these nuclei, NR3A may influence the integrating roles of these nuclei.

The paraventricular nucleus of the thalamus is a part of this midline-intralaminar complex that projects to the deep layers of the prefrontal cortex and to the shell region of the nucleus accumbens (Otake and Nakamura, 1998; Berendse and Groenewegen, 1990, 1991; Bubser and Deutch, 1998; Pinto et al., 2003). The paraventricular nucleus has been implicated in the regulation of autonomic and visceral functions (Bhatnagar and Dallman, 1999), particularly in response to stress (Jaferi et al., 2003; Bhatnagar and Dallman, 1998; Bhatnagar et al., 2000; Spencer et al., 2004), as well as behavioral responses to psychostimulant drugs (Deutch et al., 1998; Young and Deutch, 1998). Lesion studies suggest that the posterior paraventricular thalamus plays a critical role in regulating hypothalamic–pituitary–adrenal (HPA) axis function involving habituation to chronic stress (Bhatnagar et al., 2002). The paraventricular nucleus has also recently been shown to modulate the activity of neurons in the central nucleus of the amygdala in response to acute psychological stressors (Spencer et al., 2004), which may have functional significance given that the central nucleus is a major source of output projections from the amygdala, and has been directly linked to many aspects of forebrain processing of stress-related information (Akmaev et al., 2004; Pitkanen et al., 1997). Consequently, NR3A may play a critical role in regulating HPA function and stress responses. Furthermore, alterations in NR3A expression may contribute to mood disorders (Mueller and Meador-Woodruff, 2004), including major depression and posttraumatic stress disorder, which are associated with disturbances in the HPA axis (Marshall and Garakani, 2002).

4.3.2. Hypothalamus

Glutamate has been found to play an important role in neuroendocrine regulation in the hypothalamus (Brann, 1995). High densities of glutamate receptors have been found in the ventromedial and medial hypothalamic regions (Eyigor et al., 2001); consistent with this, we found pronounced expression of NR3A in the ventral medial and dorsal medial nuclei.

Evidence suggests that the dorsomedial nucleus modulates a variety of endocrine, autonomic, and metabolic functions (Koutcherov et al., 2004; Thompson and Swanson, 1998; Thompson et al., 1996). For example, functional studies in rodents implicate the dorsomedial nucleus in central regulation of food intake and metabolism, while dorsomedial nucleus neurons and perikarya contain key appetite-regulating molecules, including leptin, orexin, and melanin-concentrating hormone receptors, cocaine- and amphetamine-regulated transcripts for neuropeptide Y and neurotensin (Bellinger and Bernardis, 2002; Elmquist et al., 1999; Thompson et al., 1996; Abbott et al., 2003). Physiological studies implicate the dorsomedial nucleus in central regulation of stress and thermoregulation. There is mounting evidence of the important role the dorsomedial nucleus plays in central regulation of cardiovascular function (Kobayashi et al., 1999; Marsh et al., 2003; Fontes et al., 2001). The high level of NR3A expression in these nuclei suggests a role for this subunit in NMDA-mediated neurotransmission associated with these regions.

4.4. Medial temporal lobe

4.4.1. Hippocampus

Medial temporal lobe structures include the hippocampal region and the adjacent perirhinal, entorhinal, and parahippocampal cortices (Squire et al., 2004). NR3A is robustly expressed in the perirhinal, entorhinal, and parahippocampal cortices, as well as in the CA2 hippocampal subfield, subiculum, and dentate gyrus, suggesting it may play an important role within this system and processes involved with memory. The hippocampus receives highly processed, multimodal sensory information via two inputs from the entorhinal cortex, compares the two inputs, and sends information back to the cortex via the entorhinal cortex, and to limbic structures via direct projections. High levels of NR3A in both the entorhinal cortex and the subiculum suggest that NR3A is well situated to regulate hippocampal input/output, and thus influence hippocampal function. The majority of neurons in the hippocampus are glutamatergic pyramidal neurons. Earlier studies of NR3A expression in the rodent hippocampus reported that NR3A mRNA was primarily expressed in the CA1 subfield of the hippocampus, peaking on postnatal day 7 and subsequently declining to low levels in all subfields in the adult (Ciabarra et al., 1995; Sucher et al., 1995; Ritter et al., 2002). Low to moderate levels of NR3A protein have also been reported in the hippocampal subfields of the rat (Wong et al., 2002). In contrast, we found that NR3A is robustly expressed selectively in the CA2 subfield of the adult primate brain, which may reflect a species difference. The selective expression of NR3A in this subfield suggests that it may play a unique role involving decreased NMDA receptor activity.

4.4.2. Amygdala

The amygdala plays a major role in the acquisition and expression of fear conditioning (Sah et al., 2003). The neural basis of fear extinction (ability to adapt as situations change by learning to suppress a previously learned fear) is believed to involve connections between the medial prefrontal cortex and the amygdala (Sotres-Bayon et al., 2004). It is proposed that connections between these two regions normally allow the organism to adjust its emotional behavior when environmental circumstances change, and that some alteration in this circuitry, might underlie the inability of persons with anxiety disorders to regulate their emotions. Lesions to the medial prefrontal cortex have been shown to increase resistance to extinction (Morgan et al., 2003), suggesting decreased cortical activity may attenuate extinction. Given this, cortical expression of NR3A may influence extinction by diminishing excitatory cortical input to the amygdala.

The output region of the amygdala, the central nucleus, has been shown to control expression of fear responses (Sotres-Bayon et al., 2004). It receives information from the lateral nucleus of the amygdala either directly or by way of intra-amygdala connections, primarily the basal accessory nucleus (another site of high NR3A mRNA expression), and sends information to brain regions that control specific conditioned responses (Sah et al., 2003). The central nucleus projects to the periaqueductal gray, hypothalamus, and brainstem, which coordinate various defensive responses such as flight, defensive fight, freezing, avoidance reactions, submissive postures, tonic immobilization, hypoalgesia, and autonomic arousal (Sah et al., 2003). It is widely accepted that NMDA receptors contribute to the synaptic plasticity that underlies learning and memory in a variety of brain systems (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993), including the amygdala system underlying fear conditioning (LeDoux, 2000; Stote and Fanselow, 2004; Walker and Davis, 2002). NR3A may therefore, influence fear extinction by modulating NMDA receptor activity within the central nucleus, the primary site of NR3A expression in the amygdala.

Dopaminergic neurotransmission is also influenced by the central nucleus, and it is thought that this amygdaloid nucleus maintains control, via an indirect pathway, involving tonic activity of a population of ventral tegmental dopamine neurons that specifically regulate basal dopamine efflux in the nucleus accumbens (Phillips et al., 2003). The control of mesocorticolimbic dopamine activity by the central nucleus can influence the incentive value of the sensory property of food, and its modulation by the drive-state of the animal (Phillips et al., 2003). Expression of NR3A in this nucleus may provide another mechanism by which NR3A may regulate midbrain dopaminergic neurotransmission.

4.5. Cerebellum

While the cerebellum coordinates voluntary movement, there is accumulating evidence that it may also play a role in cognition (Kyosseva, 2004; Marien et al., 2001; Rapoport et al., 2000). It receives input from all levels of the central nervous system (Voogd, 2003; Middleton and Strick, 2000). In general, these inputs are vestibulocerebellar (governing bodily equilibrium and eye movements), spinocerebellar (controlling execution of limb movements), and cerebrocerebellar (implicated in the initiation, planning and timing of movements). The cerebrocerebellar pathway is an obvious candidate pathway when considering an anatomical basis for cerebellar–cognitive interactions. Its afferent projections are derived not only from sensorimotor cortical systems, but also include substantial contributions from dorsolateral and medial prefrontal cortices, frontal language regions, posterior inferior and superior parietal cortices, superior colliculus and superior temporal cortex. Other afferent inputs include those from anterior cingulate cortex and posterior hypothalamus, as well as major inputs from neuromodulatory noradrenergic, serotonergic and dopaminergic brainstem nuclei. High levels of NR3A mRNA in the cerebellum suggest that NR3A may modulate motor and cognitive functions referable to this region of the brain.

4.6. Conclusion

In conclusion, unlike previous reports in the rodent that NR3A expression is robust early in development but eliminated by adulthood throughout the brain, we have found relatively high levels of expression of this NMDA receptor subunit in the adult primate brain. While widely distributed, NR3A is expressed in relatively higher amounts in discrete regions, suggesting that it may have a specific functional role in defined circuits associated with these brain areas.

Appendix A. List of abbreviations

A	anterior nuclei of the thalamus
ABA	accessory basal amygdaloid nucleus
Acg	anterior cingulate gyrus
AnG	angular gyrus
Ant	anterior nuclei of thalamus
Amy	amygdala
BA	basal amygdaloid nucleus
CA1–CA2	subregions of the hippocampus
Cb	cerebellum
cc	corpus collosum
Cd	caudate
CeA	central amygdaloid nucleus
CGM	central gray substance of medulla
CGMB	central grey substance of midbrain
CIC	inferior colliculus
Cl	claustrum
CM	central medial nucleus of the thalamus
CMRt	central medullary reticular group
CnF	cuneiform nucleus

CoA	cortical amygdaloid nucleus
Cun	cuneus gyrus
DG	dentate gyrus
DH	dorsal hypothalamic area
DMH	dorsomedial nucleus of hypothalamus
DIO	dorsal accessory inferior olivary nucleus
DR	dorsal raphe nucleus
Dt	dentate nucleus of cerebellum
EmB	emboliform nucleus
Ent	entorhinal cortex
Fag	fastigial nucleus
FOG	fronto-orbital gyrus
FuG	fusiform gyrus
Gi	gigantocellular nucleus
Glo	globose nucleus
GP	globus pallidus
GRe	gyrus rectus
Hipp	hippocampus
Hyp	hypothalamus
iml	intralaminar nuclei of the thalamus
IFG	inferior frontal gyrus
Ins	insular cortex
IOG	inferior occipital gyrus
ION	inferior olivary nuclei
ITG	inferior temporal gyrus
Hyp	hypothalamus
LA	lateral amygdaloid nucleus
LBA	lateral basal nucleus
LD	lateral dorsal nucleus of thalamus
LGN	lateral geniculate nucleus
LH	lateral hypothalamus
LHb	lateral habenular
LiG	lingual gyrus
LPGi	lateral paragigantocellular nucleus
LPul	lateral pulvinar nucleus of thalamus
LVe	lateral vestibular nucleus
MD	medioldorsal nucleus of thalamus
MeA	medial amygdaloid nucleus
MFG	middle frontal gyrus
MGN	medial geniculate nucleus
MHb	medial habenular nucleus
MIO	medial accessory inferior olivary nucleus
MM	mamillary bodies
Mpul	medial pulvinar nucleus
MTG	middle temporal gyrus
MVe	medial vestibular nucleus
Nac	nucleus accumbens
Occ	occipital cortex
OG	occipital gyrus
PA	periamygdaloid area
Pcg	posterior cingulate gyrus
PCu	pre-cuneus
PCRt	parvicellular reticular nucleus
PF	parafascicular nucleus
PHR	posterior hypothalamic area
Pn	pontine nuclei
PnO	oral pontine reticular nucleus
PoG	posterior gyrus
PPHG	parahippocampal gyrus
PPTg	pedunculopontine tegmental nucleus
PrS	pre-subiculum
PrG	pre-central gyrus
Pul	pulvinar
Pu	putamen
PV	paraventricular nucleus of thalamus
Re	reuniens nucleus
Rt	reticular nucleus of thalamus
RPC	parvocellular part of red nucleus
RtTg	reticulotegmental nucleus
SFG	superior frontal gyrus
SF	subfascicular nucleus
SG	supragenicular nucleus
SMG	supramarginal gyrus
SNc	substantia nigra, pars compacta
SPL	superior parietal lobule
STG	superior temporal gyrus
STh	subthalamic nucleus
StT	nucleus of stria terminalis
Sub	subiculum
SuC	superior central nucleus
SuM	supramammillary nucleus
SuVe	superior vestibular nucleus
V	ventral nuclei of thalamus
VA	ventral anterior nucleus of thalamus
Ve	vestibular nuclei
VMG	ventral nucleus of medial geniculate body
VMH	ventromedial nucleus of hypothalamus
VTA	ventral tegmental area
ZI	zona incerta

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