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Novel regional and developmental NMDA receptor expression patterns uncovered in NR2C subunit- β -galactosidase knock-in mice

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Abstract

NMDA receptor “knock-in” mice were generated by inserting the nuclear β -galactosidase reporter at the NR2C subunit translation initiation site. Novel cell-types and dynamic patterns of NR2C expression were identified using these mice, which were unnoticed before because reagents that specifically recognize NR2C-containing receptors are non-existent. We identified a transition zone from NR2C-expressing neurons to astrocytes in an area connecting the retrosplenial cortex and hippocampus. We demonstrate that NR2C is expressed in a subset of S100 β -positive/GFAP-negative glial cells in the striatum, olfactory bulb and cerebral cortex. We also demonstrate novel areas of neuronal expression such as retrosplenial cortex, thalamus, pontine and vestibular nuclei. In addition, we show that during cerebellar development NR2C is expressed in transient caudalrostral gradients and parasagittal bands in subsets of granule cells residing in the internal granular layer, further demonstrating heterogeneity of granule neurons. These results point to novel functions of NR2C-containing NMDA receptors.

Introduction

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that play an important role in brain development, excitatory neurotransmission, and synaptic plasticity associated with learning and memory. Over-activation of NMDARs has also been implicated in excitotoxicity, as occurs during ischemia and in neurological disorders.

NMDARs are comprised of an obligatory NR1 and at least one type of NR2 subunit (NR2A - NR2D); NR3A and NR3B subunits form tri-heteromeric receptors with NR1 and NR2 subunits (Cull-Candy and Leszkiewicz, 2004; Dingledine et al., 1999; McBain and Mayer, 1994). It is critically important to understand the precise patterns of NR2 subunit expression during development because they confer to NMDARs distinct affinities for glutamate, unitary channel conductances, activation and inactivation kinetics, sensitivity to voltage-dependent magnesium block and subcellular distribution (Carroll and Zukin, 2002; Cull-Candy et al., 2001; Philpot et al., 2001; Rao and Craig, 1997; Rao et al., 1998; Wenthold et al., 2003). Based largely on *in situ* hybridization studies, and later confirmed electrophysiologically (Cathala et al., 2000)

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it is generally accepted that the properties of NMDAR currents during early neonatal development conform to NR2B- and NR2D-containing channels, and after the first postnatal week, NMDA receptors with properties of NR2A- and NR2C-containing channels are observed (Cull-Candy and Leszkiewicz, 2004). The distinct spatiotemporal patterns of NR2 subunit expression are largely regulated by transcription (Desai et al., 2002; Myers et al., 1999; Sasner and Buonanno, 1996).

While reagents have been available to study the regional and subcellular distribution of NR2A- and NR2B-containing receptors, studies of NR2C expression have been mostly restricted to mRNA analysis because NR2C antisera cross-react with NR2A/NR2B subunits and are inadequate for immunohistology (Wenzel et al., 1997). Transgenic mice expressing the β -galactosidase (β -gal) reporter under control of the NR2C promoter have also been uninformative because, as a result of insertion site variegation, mouse lines harboring identical reporter constructs show variable patterns of β -gal expression in different brain regions or ectopic sites of expression (Suchanek et al., 1997) that do not coincide with NR2C mRNA expression (Daggett et al., 1998; Goebel and Poosch, 1999; Sato et al., 1998; Sato et al., 1999); this paper). Therefore, most studies on NR2C-containing receptors have been restricted to cerebellar granule neurons.

In order to analyze the developmental, regional and cell type-specific expression of NR2C, we have used homologous recombination to knock-in a cDNA encoding nuclear β -gal ($n\beta$ -gal) into the translation initiation codon of the NR2C gene. This strategy has the advantage that transcription of *nlacZ* is driven by transcription regulatory elements in the NR2C locus (i.e., enhancer, insulators and locus control regions), and that $n\beta$ -gal translation occurs from the cognate NR2C translation initiation site. Using these NR2C^{nlacZ} knock-in mice, we have uncovered a previously unrecognized dynamic regulation of NR2C in the developing cerebellum, novel regional expression of NR2C in forebrain structures, and expression in non-neuronal cells of the brain. These findings will contribute to our understanding of NMDARs with distinct electrophysiological properties, such as magnesium-sensitivity and single-channel conductances, and the role of NMDARs in pathological responses to brain injury.

Results

Generation of NR2C^{nlacZ} knock-in mice

We used gene targeting in ES cells to generate mice in which the *E. coli nlac-Z* reporter gene was “knocked-in” into the translation initiation codon of the NR2C gene (*Grin2-C*). The targeting vector was designed to recombine with the *Grin2-C* locus to replace exons encoding the translation initiation site down to the fourth transmembrane domain (Fig. 1A). PCR analysis and Southern blots were used to confirm the accuracy of site-specific recombination. In these knock-in mice expression of the $n\beta$ -gal reporter is controlled by all the endogenous NR2C regulatory elements. In addition, histological staining for $n\beta$ -gal using X-gal is extremely sensitive, provides a three dimensional anatomical map of NR2C expression in the brain and, by virtue of its nuclear localization, it permits unambiguous identification of the precise cells expressing NR2C protein in the developing brain. The floxed pGKneo cassette was removed by crossing the homozygous NR2C^{nlacZ} mice with EIIa-Cre transgenic mice. A diagram of the resulting allele after removal of the neomycin cassette is shown in Fig. 1B. We found that removal of pGK-neo increased expression levels of $n\beta$ -gal by approximately 4-fold compared to the pGK-neo allele (data not shown). In contrast to mouse lines derived by non-homologous transgene insertion that often exhibit variegating expression patterns depending on the sites of integration, NR2C^{nlacZ} mice showed no such differences either across generations or between the 2 independent lines we generated. As discussed below, all sites of NR2C expression identified by X-gal staining of NR2C^{nlacZ} mice, hereon referred as NR2C expression, faithfully reproduced the expression patterns of endogenous NR2C transcripts as confirmed by *in situ*

hybridization (see below; Fig. 6). NR2C^{nlacZ} knock-in mouse lines are viable, reproduce normally and do not exhibit any overt behavioral alterations, consistent with previous reports of NR2C mutant mice (Ebralidze et al., 1996; Ikeda et al., 1995; Sprengel et al., 1998).

Nuclear β -gal reveals a cerebellar caudal-rostral NR2C expression gradient

The patterns of NR2C expression in different cerebellar lobules were analyzed during development using sagittal sections obtained from heterozygous NR2C^{nlacZ} mice. As shown in Figure 2, expression of NR2C proceeded in a caudal-rostral gradient along the vermis during an extended period of postnatal development. The earliest, weak X-gal staining was observed at P5, where it was restricted to the internal granule cell layer (IGL) in the gyrus of lobule IX (not shown). Between P6 and P12, NR2C expression proceeds rostrally and X-gal staining in granule cells extends from lobule IX to the mid-dorsal gyrus of lobule VII (Fig. 2A, B). Isolated cells in the vestibular nuclei began to be detected during this developmental period. By P16 expression of NR2C was evident in some, but not all, granule cells that extend from lobule I to the anterior gyrus of lobule VI, and the strongest β -gal staining continued to be observed between lobules VII and IX (Fig. 2C). Thus, with the notable exception of weak staining at the posterior edge of lobules VI and X, expression of NR2C could be detected by P16 in granule cells of all cerebellar folia. As the cerebellum matures during a period that follows granule cell migration (> P16), there is a further pronounced increase in the intensity and extent of β -gal expression throughout the entire IGL, which reaches steady-state maximal levels by approximately the second month of age (Fig. 2D). It should be noted that the pattern of NR2C expression in the vermis described above was also observed along the lateral lobes. Consistent with our previous *in situ* hybridization studies on NR2C mRNA expression (Sasner and Buonanno, 1996) and with results shown below (see Fig. 6), β -gal expression was restricted to post-migratory granule cells located in the IGL and was never observed in migrating granule neurons or in the Purkinje cell layer of the external germinal layer. Interestingly, within the IGL, we first detected β -gal expression in the areas adjacent to the Purkinje cell layer and the staining remained strongest in this region at P16 (arrows in Fig. 2C).

NR2C is expressed in cerebellar parasagittal bands during development

The functional and molecular compartmentalization of the cerebellum in transverse zones has mostly been described for Purkinje cells and their connections in the molecular layer, whereas the modular organization of granule cells has received considerably less attention (Herrup and Kuemerle, 1997; Ozol et al., 1999; Ozol and Hawkes, 1997). The paucity of these studies is due, at least in part, to the scarceness of reagents that recognize the modular organization of the IGL, as compared to markers that identify transverse parasagittal bands in the Purkinje cell layer (Ozol et al., 1999) and to the widespread perception that granule cells are a homogenous population of neurons evenly distributed throughout the IGL. Given the pronounced differences we observed in the caudal-rostral expression pattern of NR2C during development, we utilized whole mount X-gal staining of entire cerebella to investigate the medial-lateral expression of NR2C during development. As shown in Figure 3A, we initially observed a clear difference in X-gal staining along the medial-lateral axis of the vermis of lobule VI. Analysis of coronal (not shown) and horizontal (Fig. 3B) sections revealed that NR2C was transiently expressed in a series of 6 parasagittal bands in granule cells. At higher magnification, we have observed that each band appeared to be composed of two stripes of β -gal-expressing granule cells. The parasagittal bands were mostly confined to the vermis and more easily detected between P16-P20 before expression expanded throughout the entire IGL. In some cases the parasagittal bands continued to stain more strongly even in adult cerebella (not shown). As discussed below (see Discussion), the parasagittal bands observed in the heterozygous NR2C^{nlacZ} mice uncover novel boundaries in the IGL supporting a high degree of compartmentalization and possible modular function.

Developmental expression of NR2C in hindbrain and forebrain structures

Previous studies on NR2C expression and function have mostly focused on the cerebellum; however, analyses of the heterozygous and homozygous NR2C^{nlacZ} mice revealed an extensive and dynamic expression of this subunit in the forebrain. The earliest NR2C expression in the forebrain appeared at P6 in the hypothalamic area in the suprachiasmatic, pontine and reticulotegmental nuclei (Fig. 4A). Between P6 and P12 there was also a marked increase in NR2C expression in mitral cells and small cells dispersed throughout the glomerular layer (GCL) of the olfactory bulb (Fig. 4A,B). At P12-P16, weak X-gal labeling could be detected in the thalamic area and staining appeared in single cells dispersed in the hippocampus. Starting from P12, we observed strong NR2C expression in a discrete area of the retrosplenial cortex (see arrow, Fig. 4B). This pronounced expression in the retrosplenial cortex, directly adjacent to the corpus callosum and ventral to layer V of the neocortex, not only marks a novel site of NR2C expression but also identifies a group of neurons previously not described. By P16, strong expression of the NR2C β -gal reporter was apparent in most thalamic nuclei, except for the centrolateral nucleus (Fig. 4C). The adult pattern of NR2C expression in the forebrain was essentially established by P25 (Fig. 4D), with the most intense X-gal staining observed in the thalamus and the pontine nucleus, and disperse light staining in the cortex, basal ganglia and hippocampus. In the hippocampus higher cell concentration was seen in the dorsal part and very few cells in the area of the dentate gyrus (arrow in Fig. 4D).

To analyze in more detail regions that express low levels of NR2C, we utilized adult homozygous NR2C^{nlacZ} mice expressing two copies of the β -gal reporter and extended staining times. We should emphasize that, in our conditions (see Methods), all X-gal staining in NR2C^{nlacZ} mice was localized to the nucleus, which differs from background cytoplasmic staining that can arise from endogenous lysosome galactosidase and no staining was observed in brains from control mice. In whole mount stainings of homozygous NR2C^{nlacZ} mice (Fig. 5A,B), the graded expression of NR2C in the neocortex is appreciated more easily. The strong expression was observed in the mid-region of the dorsal (Fig. 5A) and ventral (Fig. 5B) cortex and the levels gradually decreased along the rostral-caudal axis with the most posterior and anterior aspects of the neocortex expressing either low or undetectable levels of NR2C (Fig. 5A,B). The strong expression of NR2C in the piriform cortex, anterior olfactory nucleus and the pontine nucleus was evident from the ventral view. Staining in the olfactory bulb is discussed below.

Next, we analyzed a series of horizontal 200 μ M free-floating sections cut from the dorsal to mid-ventral region (Fig. C–H). The most dorsal sections emphasize the rostral-caudal extent of NR2C expression in the retrosplenial cortex, as well as its broad expression throughout the dorsal hippocampus (Fig. 5C,D). There was a complete absence of X-gal staining in the white tracts of the corpus callosum and superior colliculus. Interestingly, while there have not been studies of NR2C-containing NMDARs in thalamus, this is the highest β -gal expressing area in the forebrain (Fig. 5E–G). Within the thalamus, the strongest expression was observed in reticular, medial (mediodorsal central, mediodorsal lateral) and ventral (ventral posterolateral, ventral posteromedial) nuclei. Reporter expression in the laterodorsal, posterior, and centrolateral thalamic nuclei was relatively low (Fig. 5E–G). In the forebrain we found numerous dispersed NR2C expressing cells in the caudate-putamen, piriform cortex, neocortex, and hippocampus. Staining in the hippocampal formation was graded, with strongest NR2C expression in the dorsal relative to the ventral side (compare Fig. 5E to 5G). In the most ventral sections strong expression was observed in the vestibular, cochlear and cuneate nuclei, and rostrally, dispersed β -gal expressing cells were seen in the perirhinal and piriform cortex (Fig. 5G,H). The olfactory bulb also expresses NR2C. The strongest X-gal staining was found along a band that coincides with mitral cells, a weaker staining region in the periglomerular layer and sparsely dispersed cells in the granular cell layer (Fig. 5G,H).

Because of the lack of information of NR2C expression in the forebrain, there have not been many studies that have reported or considered the presence of NR2C-containing NMDARs in this area of the brain.

NR2C mRNA expression during brain development

To confirm that n β -gal expression faithfully reflected expression of the NR2C gene, *in situ* hybridization experiments were performed to analyze endogenous transcripts in thin paraffin sections from fetal (E14–18), postnatal (P0–P16), and adult (P25–28, 1 year) mouse brains. We utilized ³³P-labeled cRNA probes in our experiments to increase sensitivity and facilitate the identification of cells expressing low levels of NR2C mRNAs. The specificity of ³³P-labeled cRNA signals was verified in brain sections prepared from homozygous NR2C^{nlacZ} mutant mice using probes corresponding to the region deleted in the knock-in allele (not shown). Special care was used to include sections from areas that previously were not reported to express NR2C, such as the retrosplenial cortex, caudate putamen and hippocampus.

No specific signals for NR2C transcripts were detected in brains from newborn mice (not shown). By P5, the most pronounced expression was observed in the forming vestibular nuclei, the olfactory bulb and a thin layer of cells in the cerebral cortex adjacent to the corpus callosum (Fig. 6A). In the cerebellum, NR2C transcripts were restricted to the posterior lobule X. The sites of NR2C mRNA expression were similar and consistent with those observed using X-gal-stained tissues from NR2C^{nlacZ} mice. In addition to the regions described above, by P8 we observed hybridization to transcripts located over the nuclei of dispersed cells throughout the hippocampus (Fig. 6B). This hybridization signal was neither to hippocampal pyramidal neurons nor to dentate granule cells. At P8 there also was strong hybridization to cells in the pontine nucleus and retrosplenial cortex, and was stronger than at P5. In the olfactory bulb NR2C mRNAs were detected in mitral cells and cells dispersed in the glomerular layer. In the cerebellum expression of NR2C extended from lobule X to the caudal half of gyrus of VII (Fig. 6B); the rostral boundary of NR2C expression at mid-lobule VII was similar to that observed in the NR2C^{nlacZ} mice (Fig. 2).

The first NR2C-positive hybridizing cells appear in the thalamus at P10 (Fig. 6C). In the cerebellum most of the lobules were positive for NR2C transcripts at this age, although there was significant heterogeneity in the intensity and pattern of expression in the IGL, similar to n β -gal expression at P16 (compare to Fig. 5C). At P10 hybridization in the cerebellum was weakest at the gyrus of lobule VI and, as in the case for NR2C^{nlacZ} mice, granule cells across the width of the IGL of lobules I–V differentially expressed NR2C transcripts. Between P12 and P16, NR2C mRNA expression reached adult levels in the thalamus and cerebellum. In addition, expression of NR2C transcripts in cells dispersed throughout the neocortex and basal ganglia became evident at this age (Fig. 6D,E) and persisted at high levels at P25 (Fig. 6F) and in adult mice (not shown).

We conclude that all n β -gal-positive regions in NR2C^{nlacZ} mice express the NR2C transcripts, confirming that these knock-in mice faithfully recapitulate the regional expression patterns of the gene and do not express the reporter gene ectopically as reported for NR2C-promoter transgenic mice (Suchanek et al., 1997). As has been reported for the NR2 subunits in rat brain (Wenzel et al., 1997), we found that in most areas NR2C mRNA expression preceded X-gal staining by 2–6 days that could be expected because one method measures RNA levels while the other requires the accumulation of protein, respectively. The only significant temporal difference observed between *in situ* hybridization and reporter levels in NR2C^{nlacZ} mice was in lobule X of the cerebellum (about 18 days). At this time we do not know the cause of this difference.

NR2C is expressed in neurons and non-neuronal cells

Numerous studies have reported AMPA receptor expression in neurons, as well as in glia (Burnashev et al., 1992; Iino et al., 2001; Steinhauser and Gallo, 1996). In contrast, until very recently, it has generally been accepted that NMDAR NR2 subunit expression is restricted to neurons (see Discussion). However, the dispersed distribution of NR2C-expressing cells in NR2C^{nlacZ} knock-in mice and the confirmation of this expression by *in situ* hybridization in the hippocampus, olfactory bulb and basal ganglia, made us ask if NR2C might be expressed by non-neuronal cells. Taking advantage of the cellular resolution afforded by the n β -gal reporter, which is restricted to the nuclei of NR2C transcribing cells, we used double immunofluorescence histochemistry with antibody markers for neurons, astrocytes and oligodendrocytes to identify the cell types that express the NR2C subunit in brain. Free-floating sections were incubated with antibodies against β -gal, in combination with antibodies against either the neuronal marker NeuN, astrocyte marker GFAP, oligodendrocyte marker CNPase, neuroglia and oligodendrocyte marker NG2, and S100 β as a general glial marker.

As shown in Figure 7, the great majority of cells in the defined group of cells in retrosplenial cortex (arrow in Fig. 7A) were NeuN positive indicating that they are of neuronal origin (Fig. 7B–D). This is the first demonstration that a distinct group of neurons in the neocortex can express the NR2C subunit. All NR2C positive cells in the vestibular nuclei, thalamic nuclei, the pontine area and cerebellum showed immunoreactivity with NeuN (Fig. 7E–M). Interestingly, in the cerebellum we found many NeuN/ β -gal-positive cells in the molecular layer that by size and morphology of the nucleus were similar to the granule cells in the IGL (Fig. 7K–M).

The “stream” of n β -gal expressing cells in the presubiculum and subiculum that connect the NR2C positive neurons in the retrosplenial cortex to the hippocampus consists mainly of non-neuronal cells, as identified by S100 β staining (Fig 8B–D). All n β -gal expressing cells that extend into the dorsal hippocampus proper (CA1–CA3) are positive for GFAP and/or S100 β , but never positive for NeuN (Fig. 8E–J), thus identifying a population of NR2C-positive astrocytes. Since assembly of functional NMDARs requires the NR1 subunit, we tested if n β -gal positive glial cells in the hippocampus are positive for NR1 immunoreactivity. As shown in Figure 8K–M, we found that cells positive for NR1 subunit are not positive for n β -gal, suggesting that glial cells expressing the NR2C subunit fail to assemble functional NMDARs. At this time we do not know if activated glial cells have the potential to express functional NMDARs (see Discussion), as suggested by prior studies (Ahlemeyer et al., 2002).

Next, we used double immunofluorescence to identify the cell types that express the n β -gal reporter in the cerebral cortex, basal ganglia and glomerular layer of the olfactory bulb of NR2C^{nlacZ} mice, and which also appeared positive for NR2C transcripts when analyzed by *in situ* hybridization (see Fig. 6). To our surprise we found that n β -gal-positive cells in all areas of the cerebral cortex (other than the defined area in the retrosplenial cortex described above), as well as cells in the caudate-putamen are negative for NeuN, suggesting they are not mature neurons. These cells also tend to have small and often elongated nuclei, as compared to neuronal nuclei labeled by NeuN. Consistent with this observation, we found that these n β -gal-positive cells were also positive for S100 β , usually considered to be a general non-neuronal marker (Fig. 9A–F). However, unlike n β -gal-positive cells in the hippocampus, these cells were not immunoreactive for GFAP. We could not find any β -gal positive cells in the white matter tracts of the corpus callosum, and the oligodendrocyte marker CNPase never co-localized with β -gal-positive cells (Fig. 9G–I).

NR2C expression in the olfactory bulb

Another area that showed strong expression of n β -gal is the olfactory bulb, in particular mitral cells that are easily identifiable by their position and size (Fig. 10A). In addition, we also observed abundant X-gal staining in the glomerular layer (arrow heads in figure 10A) and in small number of cells in the granular layer. Double labeling showed that most of the n β -gal-positive cells of the glomerular layer and all cells in the granular layer were NeuN negative (Fig. 10B–D). However, at a higher magnification in the confocal microscope we could detect some n β -gal-positive periglomerular cells that had very low levels of NeuN expression (arrows in Fig. 10E–G). The periglomerular layer in the olfactory bulb has been shown to contain 3 types of interneurons: type 1 are γ -aminobutyric acid (GABA) positive, type 2 express Ca-binding proteins calretinin and calbindin, and type 3 are immunoreactive for the neuropeptides somatostatin or cholecystokinin (CCK) (Gutierrez-Mecinas et al., 2005). We used GABA (Fig. 10H–J), CCK (Fig. 10K–M), and calretinin (Fig. 10N–P) antibodies in double immunostainings to further characterize the n β -gal-positive PG cells. However, none of the used markers co-localized with the n β -gal-positive cells.

To determine if these cells are of glial origin, we double-labeled sections with S100 β , GFAP, NG2 and CNPase antibodies. We never found S100 β in the n β -gal-positive periglomerular cells (Fig. 10Q–S), though this marker co-localized with the sparsely dispersed cells in the granular layer (data not shown). Likewise, none of the n β -gal-positive cells in the olfactory bulb were positive for GFAP (data not shown), the neuroglial/oligodendrocyte marker NG2 or the oligodendrocyte marker CNPase (Fig. 10T–Y). Therefore, this n β -gal-positive cells in the olfactory bulb represent a group of cells of undetermined lineage as defined by available cell-type markers.

Discussion

In agreement with previous reports (Ebrilidze et al., 1996; Ikeda et al., 1995; Kadotani et al., 1996), NR2C^{nlacZ} null mice do not exhibit obvious altered behaviors. However, NMDAR-EPSC peak amplitudes and charge transfer are significantly increased at autaptic synapses of cerebellar granule cells isolated from NR2C^{nlacZ} null mice (Lu et al., 2006), consistent with previous studies performed in slices from NR2 null mice (Ebrilidze et al., 1996; Takahashi et al., 1996). While previous reports on NR2C have focused on cerebellar function, we have used NR2C^{nlacZ} knock-in mice to accurately determine, with cellular resolution, the spatiotemporal expression of NR2C throughout the brain. Our findings showing NR2C expression in other neuronal and glial populations should expand the future scope of functional and behavioral experiments.

Heterogeneity of cerebellar granule neurons

The cerebellum is a highly compartmentalized structure, where transverse and sagittal modules are defined by function, innervation patterns and molecular markers (Armstrong and Hawkes, 2000; Herrup and Kuemerle, 1997). While molecular markers that identify distinct Purkinje cells within lobules are numerous, fewer markers define granule cell heterogeneity. Our analysis of developing NR2C^{nlacZ} mice revealed three new spatiotemporal expression patterns within the IGL.

Rostrocaudal gradient—The cerebellum is proposed to be subdivided rostrocaudally into 4 transverse zones (Herrup and Kuemerle, 1997; Ozol et al., 1999), denoted as the anterior (lobules I–V), central (~ lobules VI–VII), posterior (~ lobule VIII to the dorsal part of IX) and nodular (~ ventral lobule IX to X) zones. We found that between P6–P12 NR2C-n β gal expression initially is confined to the posterior zone, followed by expression in the anterior (P12–P16) and central (after P16) zones, and after a long delay, n β -gal activity is observed in

the nodular zone. Hence, the graded expression of NR2C in IGL neurons follows a pattern similar, but distinct, to other rostrocaudal markers expressed by Purkinje and/or EGL neurons (Hawkes and Gravel, 1991; Herrup and Kuemerle, 1997). Although temporarily distinct, the rostrocaudal expression patterns of GABAR $\alpha 6$ (Mellor et al., 1998) and NR2C β -gal knock-in mice are the most similar, and both genes differ from other cerebellar transverse markers in that their early expression is restricted to IGL neurons (Herrup and Kuemerle, 1997; Ozol and Hawkes, 1997). Because expression of NR2C and GABAR $\alpha 6$ are temporarily distinct, it is unlikely that the factors regulating their graded expression are related to the timing of granule cell migration or mossy fiber innervation. Analyses of mutant mice exhibiting lobulation defects, suggest cues regulating future rostrocaudal boundaries that originate during prenatal development.

Parasagittal bands—The mediolateral compartmentalization of the cerebellar Purkinje cell layer is well documented, especially the characterization of Zebrin II-positive parasagittal bands (Brochu et al., 1990). More recently, parasagittal compartmentalization during fetal and postnatal development of the granule cell layer was reported based on studies using a variety of markers, such as NADPHd, acetylcholinesterase, cytochrome oxidase and synaptophysin (Ozol and Hawkes, 1997). Expression of these markers usually is transient and spans both the granular and molecular cell layers, often aligning with Zebrin positive bands. The spatiotemporal expression of NR2C-n β -gal differs from previously studied markers. NR2C is expressed in 6 parasagittal bands, each composed of 2 stripes, confined mostly to the vermal IGL of lobule VI. While the functional significance of parasagittal bands is unknown, Purkinje cell compartments have been proposed to segregate afferent projections that later give rise to functional maps in distinct cerebellar layers (Hawkes and Gravel, 1991; Sotelo and Wassef, 1991).

Dorsal-ventral IGL regions—The third type of heterogeneity is observed in the anterior zone, where NR2C expression in the IGL adjacent to the Purkinje cell layer precedes expression in neurons closer to the white matter (see Fig. 2). Intriguingly, expression of NR2C-n β gal in the superficial aspect of the IGL is associated with the late generation of granule cells migrating from the EGL, a pattern that is opposite to that observed in GABAR $\alpha 6$ knock-in mice (Mellor et al., 1998). NR2C-n β gal mice should serve as an invaluable tool to determine the role of genetic and epigenetic factors in compartmentalizing the cerebellum.

Neuronal expression of NR2C in non-cerebellar neurons

Studies on NR2C function have been largely confined to cerebellar granule cells because their location and morphology facilitate identification for electrophysiological characterization. We have used the NR2C^{n β lacZ} mice to investigate other NR2C-expressing cells where identification is more difficult like the retrosplenial cortex, pontine, vestibular and thalamic nuclei. Using *in situ* hybridization and double immunofluorescence, we have confirmed that these NR2C-expressing cells are neurons. These findings are especially important given the lack of antibodies, specific pharmacological tools and electrophysiological properties that distinguish NR2C-containing receptors. Although lower sensitivity to magnesium block and low channel conductance are frequently used to identify NMDARs as NR2C-containing, these are not properties uniquely conferred by the NR2C subunit (Misra et al., 2000; Sasaki et al., 2002). Therefore, knowing the precise patterns of NR2 and NR3 subunit expression is of major importance when considering if an NMDA channel is NR2C-containing, as recently reported for layer IV spiny stellate cells of the mouse barrel cortex (Binshok et al., 2006).

The identification of novel NR2C-expressing neurons outside of the cerebellum, and the capacity to record from the NR2C^{n β lacZ} mutant mice, should help elucidate the specialized functions that NR2C-containing receptors may serve in the brain. This is especially interesting

in thalamic neurons, considering the role of NMDARs in schizophrenia (Javitt and Zukin, 1991), and, more specifically, NR2C subunit which is selectively reduced in this structure in postmortem samples of persons diagnosed with schizophrenia (Meador-Woodruff et al., 2003). Since Neuregulin-1 regulates NR2C mRNA levels in cerebellar slice cultures (Ozaki et al., 1997) and has been identified as a risk gene for schizophrenia (Law et al., 2006; Stefansson et al., 2003), this raises a potentially interesting link.

Expression of the NR2C in non-neuronal cells

We observed dispersed X-gal-stained cells throughout the dorsal hippocampus, subiculum, striatum and neocortex (especially the perirhinal cortex) in a pattern that corresponded to our, and others, *in situ* hybridization studies of NR2C mRNAs (Daggett et al., 1998; Watanabe et al., 1993). These other groups suggested possible glial expression of NR2C based solely on the size of hybridizing cells. In our study we show these cells indeed are not of neuronal origin because they are NeuN negative and S100 β positive. Double immunofluorescence labeling confirmed that the NR2C-n β -gal expressing cells are GFAP-positive (astrocytes) only in the hippocampus, while in other areas they were negative for microglia, neuroglia and oligodendrocytes markers.

At this time we are uncertain of the possible functional significance of NR2C expression in hippocampal astrocytes. The fact that we were not able to immunologically detect the NR1 subunit in these cells suggests that, at rest, astrocytes lack the capacity to express functional NMDAR channels or that NR1 levels are below detection. An intriguing possibility is that NR1 expression is upregulated in hippocampal astrocytes by ischemia or anoxia, as previously reported (Krebs et al., 2003). Consistent with the idea that functional NR2C-containing NMDARs may be expressed in response to ischemia, cerebral vascular occlusion selectively increases NR2C mRNA expression in vulnerable areas (Small et al., 1997) and mutation of the NR2C gene reduces ischemic injury in mice (Kadotani et al., 1996). Interestingly, we observed that NR2C-n β -gal expression is highest in CA1 and subiculum, as compared to the dentate, which coincides with regions most susceptible to global ischemic damage.

Oligodendrocytes and their precursors have long been known to express non-NMDA glutamate receptors (Gallo and Ghiani, 2000). Hypoxic/ischemic damage to oligodendrocytes had been attributed to their expression of calcium-permeable AMPA receptors, but 3 recent studies suggest that NMDARs are expressed in oligodendrocytes and contribute to myelin damage following hypoxia (Karadottir et al., 2005; Micu et al., 2006; Salter and Fern, 2005). Based on immunofluorescence and Western blot experiments with commercial antisera and electrophysiological recordings from oligodendrocytes displaying NMDARs with reduced magnesium sensitivity the reports conclude that oligodendrocytes express NR2C- and/or NR2D-containing NMDARs. However, we failed to detect X-gal staining or immunoreactivity in CNPase-positive cells in myelinated tracts of the corpus callosum, cerebellum or other areas, and we are not aware of *in situ* hybridization studies reporting NR2C expression in oligodendrocytes. The lack of NR2C-n β -gal expression in oligodendrocytes is consistent with earlier studies that failed to detect NMDAR expression in the optic nerve (Matute et al., 1997) and cultured oligodendrocytes (Patneau et al., 1994). However, electrophysiology is a very sensitive method and we cannot exclude the possibility that NR2C expression is either transient and can be activated by anoxia, or is below the detection levels in these cells.

In summary, we have uncovered a dynamic spatiotemporal pattern of NR2C expression in the developing cerebellum, and identified novel sites and cells that express the subunit. Based on these findings, novel functions of NR2C-containing NMDARs may be identified in neuronal and non-neuronal cells, which could have important implications for development and hypoxic/ischemic damage.

Experimental Methods

Generation of NR2C^{nlacZ} knock-in mice

The homologous recombination construct harboring the *E. coli nlacZ* gene cassette encoding nuclear-targeted β -gal (n β -gal) was designed to remove the first 11 coding exons of the NR2C gene (symbol: GRINC) and to insert the reporter at the initiator methionine codon (Fig. 1A). A neomycin cassette was used for positive selection of transfected embryonic stem (ES) cells with G418, and a thymidine kinase cassette used for negative selection with gancyclovir. The pGK-neo cassette was flanked by loxP sequences for its subsequent removal by crossing the homozygous NR2C^{nlacZ} mice with EIIa-Cre transgenic mice to avoid potential interference with n β -gal reporter expression (Lakso et al., 1996). TC-1 (129Sv/Ev derived) ES cells were transfected by electroporation, and resultant clones were screened by Southern blotting for homologous recombination of left and right arms. HindIII digests were used for the left arm probe (bar marked LAP, 1.5 kb in Fig. 1A), and EcoRV digests for the right arm probe (RAP, 4.1 kb) (Fig. 1C). Two independent ES cell clones were selected for microinjection into C57BL/6 blastocysts. Founder mice were screened by Southern analysis with the right arm probe (Fig. 1D). In later generations, tail genotyping was done by PCR with primers: 5'-CTATCGCCTTCTTGACGAGTTCTTC-3', and 5'-TGGTCGAACGCAGACGCGTGTGA-3'. All mouse procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Histochemical detection of n β -gal in tissue

Postnatal and adult brains from control, NR2C^{nlacZ} heterozygous and homozygous mice were used. Unfixed sagittal, coronal and horizontal brain slices (~100–300 μ m thick) were made with a vibratome (Vibratome 1000 Plus, Warner Instruments). Slices were fixed for 30 min at 4°C in 4% paraformaldehyde (PFA) buffered with 0.1M phosphate buffer (pH = 7.5), washed for 1 hour in 0.1M phosphate buffer containing 2mM MgCl₂, 0.01% deoxycholate, and 0.02% NP40, and incubated overnight at 30°C with 0.1% X-gal (5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside) in the same buffer solution. Slices from wild-type mice were used as controls for β -gal background staining. After removing X-gal by multiple washes in PBS (pH 7.4), slices were kept in 4% PFA at 4°C. Images were collected on a Nikon SMZ-U dissecting microscope (0.75–3 zoom) and a Sony DKC-5000 digital camera.

Antibodies and immunohistochemistry

Adult brains from NR2C^{nlacZ} heterozygous mice were fixed by cardiac perfusion with 4% PFA in PBS, followed by overnight fixation in 4% PFA in PBS at 4°C. Brains were then incubated for 24h in 10% glycerol, 4% PFA in PBS, and kept for at least 24h in 20% glycerol in PBS (4°C) before freezing and sectioning on a Cryo-microtome (Microm HM430). Free-floating sections (50 μ m) were incubated in blocking solution (20% goat serum, 1% BSA, 0.25% Triton X-100 in PBS) for 2 hours at room temperature. Mouse monoclonal (Promega Corporation) or rabbit polyclonal (Cortex Biochem) antibodies against β -gal were used in combination with antibodies against polyclonal GFAP (Diasorin), polyclonal S100 β (Research Diagnostics), monoclonal NeuN (Chemicon International, Inc), monoclonal NR1 (Upstate Biotechnologies), polyclonal cholecystokinin (CCK) (Chemicon International, Inc), monoclonal CNPase (Sigma), polyclonal GABA (Sigma), polyclonal NG2 (Chemicon International, Inc), and monoclonal calretinin (NeoMarkers, Fremont, CA). All primary antibodies were diluted in PBS with 1% goat serum, 1% BSA, 0.25% Triton X-100. Secondary antibodies conjugated to Alexa488 and Alexa594 (Jackson Labs) were used at a 1:500 dilution. Images were obtained on a visible/ultraviolet laser-confocal microscope (Zeiss LSM 510 inverted; objectives 40X and 63X oil). One micron optical sections were acquired using LSM 510 Meta software and imported into Adobe Photoshop 6.0.

In situ hybridization histochemistry

Littermates obtained from breedings between heterozygote NR2C^{nlacZ} mice were used for *in situ* hybridization histochemistry studies. Brains from NR2C^{nlacZ} and wild type control (littermates) mice older than postnatal day 16 (P16) were fixed by cardiac perfusion with 4% PFA in PBS. Brains were fixed in 4% PFA in PBS for 24 hours (4°C). Tissues were dehydrated, embedded in paraffin and sectioned (10 µm). *In situ* hybridization was performed with a ³³P-radiolabeled 808 bp cRNA probe spanning the sequence between the translation initiation codon and the first transmembrane domain (802bp–1610 bp generated by RT-PCR with primers: 5'TCTCACTTCACTCCTTGGTGC3' and 5'CTCGGTGACCACACTGATGA3'). Hybridization was done essentially as described elsewhere (Wilkinson and Nieto, 1993). Sections were exposed to Kodak NTB 2 emulsion for a week, counterstained with hematoxylin, and visualized by dark-field microscopy.

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Figure 1.

Targeting strategy to generate *NR2C^{nlacZ}* mice. **A: Top:** Diagram showing targeted segment of the endogenous *NR2C* locus. Lines represent introns, open boxes upstream noncoding exons, dark grey boxes coding exons. Numbers refer to transmembrane domains 1–4. Positions of left- and right-arm probes (LAP, RAP) used for Southern screening are indicated on top. **Bottom:** *NR2C* - β -gal targeting vector containing homology arms, the *lacZ* gene with nuclear localization signal (*Nuc lacZ*) inserted in-frame with endogenous AUG codon, and the pGK-neo cassette flanked by loxP sites (black boxes). **B:** Rearranged *NR2C^{nlacZ}* allele in which the *lacZ* gene replaced the first 11 exons of the *NR2C* gene and the pGK-neo cassette was excised by crossing *NR2C^{nlacZ}* mice with *EIIa-Cre* transgenic mice. **C:** Southern blots of ES cell genomic DNA, hybridized with the left arm (LAP) and right arm (RAP) probes as indicated (see A). For the left arm the HindIII digest generates a 5.7 kb wild type and 8.7 kb homologously recombined band (arrow). For the right arm (EcoRV digest) a wild type band is 8.0 kb, and a recombined band is 6.5 kb (arrow). **D:** Southern blot of tail genomic DNA from wild types (+/+), homozygotes (-/-), and heterozygotes (-/+), hybridized with the right arm probe.

Figure 2.

NR2C is expressed in a caudal-rostral gradient in the developing cerebellum. Parasagittal cerebellar slices from P6 (A), P12 (B), P16 (C), and P25 (D) heterozygous *NR2C^{nlacZ}* mice stained with X-gal. Arrows in (C) indicate stronger expression in granular cells adjacent to Purkinje cell layer in IGL. Vn – vestibular nuclei. Scale bar 2 mm.

Figure 3.

Parasagittal bands of NR2C expression in the developing cerebellum. Whole mount (A) and horizontal section (B) of heterozygous NR2C^{nlacZ} cerebelli stained with X-gal reveal 6 bands in the vermal area. **A:** At P16, the caudal part of lobule VI, as well as lobule V, are not stained, while the bands (arrows) are most prominent in the rostral part of lobule VI. **B:** At P20, parasagittal bands of X-gal staining are readily appreciated across lobules II–VII in the vermal area.

Figure 4.

Developmental changes of NR2C expression as demonstrated in X-gal stained parasagittal forebrain slices of P6–P25 (A–D) heterozygous NR2C^{nlacZ} mice. The arrow in (B) indicates a stained group of cells in the retrosplenial cortex. Note the absence of expression in the centrolateral thalamic nucleus at P16 (arrow) in (C). At P25, the adult pattern of NR2C expression is established. The arrow in (D) indicates the absence of expression in dentate gyrus. OB - olfactory bulb; SCn – suprachiasmatic nucleus; RtTg – reticulotegmental nucleus; RSC – retrosplenial cortex; Po – pontine; H – hippocampus; CLn – centrolateral thalamic nucleus; cc – corpus callosum. Scale bar 2mm.

Figure 5.

NR2C is broadly expressed in the adult brain. **A–B:** Whole mount X-gal staining of P28 brain from homozygous NR2C^{lacZ} mice shown from dorsal (A) and ventral (B) side. The arrow in (A) marks the defined band of expression in the retrosplenial cortex. There is wide spread expression in the cerebral cortex, especially on the ventral side in the anterior olfactory nuclei and the piriform cortex. **C–H:** Horizontal slices from adult homozygous NR2C^{lacZ} mice stained with X-gal. Dorsal sections in (C, D) show strong expression in retrosplenial cortex (arrow), hippocampus and presubiculum. Note the absence of expression in white tracts of the corpus callosum (arrowhead). **E–F:** In the forebrain, highest expression is seen in multiple thalamic nuclei, and lower levels in caudate-putamen, piriform and perirhinal cortex. **G–H:** In

ventral sections strong expression is apparent in the vestibular, cochlear and cuneate nuclei. Rostrally, dispersed NR2C expressing cells are seen in the piriform cortex. Mitral and periglomerular cells in the olfactory bulb are strongly stained. Staining in the granular layer is very low because n β -gal positive cells are very sparse in this layer. OB – olfactory bulb; AON- anterior olfactory nuclei; RSC – retrosplenial cortex; Pir – piriform cortex; Po – pontine; H – hippocampus; PrS – presubiculum; cc – corpus callosum; CPu – caudate putamen; PoT – posterior thalamic nuclear group; Cb – cerebellum; VPL – ventral posterolateral thalamic nucleus; VPM – ventral posteromedial thalamic nucleus; Vn – vestibular nuclei; CoN- cochlear nuclei.

Figure 6.

In situ hybridization confirms regional NR2C expression pattern identified by n β -gal reporter in NR2C^{nlacZ} mice. **A–F:** NR2C mRNA expression in thin sections of developing brains (P5–P28) from heterozygous NR2C^{nlacZ} mice. **A:** At P5, strongest expression is seen in the olfactory bulb and in the cerebellum in lobule X (arrow). Note the strong signals in the retrosplenial cortex and the lack of expression in corpus callosum in (A–C). Sparse labeling is discernable starting from P12 in the neocortex (D–F), hippocampus (B–D), and late in the caudate putamen starting from P16 (E–F). OB – olfactory bulb; RSC – retrosplenial cortex cc – corpus callosum; H – hippocampus; Th – thalamus; Vn – vestibular nuclei; Po – pontine. Scale bar 2mm.

Figure 7.

NR2C is expressed in forebrain neurons. **A:** Coronal section from heterozygous NR2C^{nlacZ} mouse stained with X-gal. The arrow identifies an area in retrosplenial cortex analyzed by double immunolabeling in (B–D). **B–M:** 50 μm sections from NR2C^{nlacZ} mice double immunolabeled with an anti- nβ-gal antibody and the neuronal marker NeuN. **B–D:** The great majority of cells in retrosplenial cortex co-express nβ-gal and NeuN. However, note that some nβ-gal positive cells (arrowheads) are NeuN negative. NeuN co-localizes with nβ-gal positive cells in vestibular nuclei (E–G), thalamus (H–J), and cerebellum (K–M). In cerebellum NeuN is co-expressed with nβ-gal not only in granule cells of the IGL but also in the dispersed cells in the molecular layer (arrows). Scale bar 20μm.

Figure 8.

NR2C is expressed in astrocytes in the presubiculum and hippocampus. **A:** Coronal sections from heterozygous NR2C^{nlacZ} mouse stained with X-gal. Arrow identifies the area in the presubiculum analyzed by double immunolabeling in (B–D); the rectangle outlines the hippocampal area shown in (E–M). Note a gradient of X-gal expression from the dorsal to ventral hippocampus. **B–D:** Most of the nβ-gal positive cells in presubiculum are NeuN negative (arrows). Note the absence of nβ-gal positive cells in corpus callosum on the left. Co-localization of nβ-gal with GFAP (E–G) and S100β (H–J) in hippocampus identifies these cells as astrocytes. **K–L:** Lack of co-localization of nβ-gal reporter and NR1 in hippocampal astrocytes. Scale bar: 20 μM.

Figure 9.

Glial expression of NR2C in neocortex and striatum. In sections from homozygous NR2C^{nlacZ} mice nβ-gal and general glial marker S100β co-localize (arrows) in the piriform cortex (A–C) and striatum (D–F). The oligodendrocyte marker CNPase does not co-localize with nβ-gal-expressing cells in the piriform cortex (G–I). Arrows identify cell bodies of CNPase positive cells. Scale bar 20 μm.

Figure 10.

NR2C is expressed in neurons and glia in the olfactory bulb. **A:** X-gal-stained coronal section of the adult olfactory bulb from homozygous NR2C^{nlacZ} mouse. Strong staining is seen in large mitral cells (MC) (arrows), and in periglomerular cells in glomerular layer (GL) (arrowheads). Few positive cells can be seen in the granular cell layer (GCL). **B–Y:** Double immuno-labeling of cells in the glomerular layer of the olfactory bulb in sections from NR2C^{nlacZ} mice. Most nβ-gal positive cells (arrows) do not co-localize with NeuN in the GL (**B–D**), except for some that are weakly NeuN-positive (**E–G**). Periglomerular interneuronal markers GABA (**H–J**), CCK (**K–M**), or calretinin (**N–P**), do not co-localize with the nβ-gal positive cells. Note the similarity in size and location between nβ-gal positive and calretinin positive cells in (**N–P**).

The general non-neuronal marker S100 β (Q–S), neuroglial/oligodendrocyte marker NG2 (T–V), or oligodendrocyte marker CNPase (W–Y) also show no co-localization with n β -gal positive cells in the glomerular layer. Scale bar: 20 μ M.