Distribution of an NMDA receptor:GFP fusion protein in sensory neurons is altered by a C-terminal construct

Daniel R. Marsh,* Kevin D. Holmes,‡ Gregory A. Dekaban†‡ and Lynne C. Weaver*

*Neurodegeneration and †Gene Therapy and Molecular Virology Research Groups, The John P. Robarts Research Institute, London, Ontario, Canada
‡Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada

Abstract

The NMDA receptor plays an important role in mediating sensory input to the spinal cord. Domains within the C-terminus of the NMDA receptor bind to cytoskeletal proteins and facilitate membrane targeting and synaptic clustering, and may participate in regulation of receptor function. One strategy to manipulate NMDA receptor function is to express C-terminal constructs in neurons to disrupt synaptic clustering via competition for binding motifs in cytoskeletal proteins and postsynaptic densities. Biolistic particle-mediated gene transfer was used to deliver plasmid DNA into organotypic cultures of dorsal root ganglia (DRG). Fusion proteins consisting of recombinant (r)NMDA receptor subunit 1-1 (rNR1-1) deletion constructs and enhanced green fluorescent protein (GFP) were expressed in sensory neurons and demonstrated unique distribution patterns within the cell. Expression of the full length rNR1-1:GFP construct was cytosolic and localized to membranous patches similar to endogenous NR1-1 protein expression in sensory neurons. Expression of a construct containing only the C-terminus, GFP:C0C1C2, demonstrated nuclear and membranous localization. When the GFP:C0C1C2 construct was co-expressed with rNR1-1 in sensory neurons, membranous localization of rNR1-1 was disrupted. In contrast, co-expression of a C-terminal cassette lacking the C1 exon cassette, GFP:C0C2, with rNR1-1 did not alter the membranous distribution of rNR1-1. This observation verifies the utility of a gene transfer strategy to diminish membranous NR1-1 content by expressing a construct containing the C1 exon cassette.

Keywords: biolistic particle, cytoskeleton, gene transfer, organotypic culture.


The targeting, clustering and anchoring of neurotransmitter receptors to appropriate postsynaptic densities are principal requirements for the formation of functional synapses (Ehlers et al. 1996a). The C-terminus of membrane receptors contains somatodendritic targeting motifs (Ehlers et al. 1995; West et al. 1997; Okabe et al. 1999), as well as elements that bind to cytoskeletal proteins and facilitate clustering and anchoring of the receptor to the membrane (Sheng 1996; Ehlers et al. 1998). The C-terminus of subunit 1 of the NMDA receptor splice variant 1a (NR1-1a) contains two exon cassettes, C1 and C2, as well as a C0 cassette that is closest to the N-terminus and is common to each of the eight NR1 splice variants. The C0 cassette interacts with α-actinin-2, an actin-binding protein (Wyszynski et al. 1997; Allison et al. 1998) that may contribute to clustering at postsynaptic sites. The C1 exon cassette is contained only in the NR1-1 and NR1-3 splice variants and is recognized as a site of significant regulation of NMDA receptor function. The C1 cassette contains phosphorylation sites for protein kinase C (Tingley et al. 1993) and protein kinase A (Tingley et al. 1997), as well as a high-affinity binding site for calmodulin (Ehlers et al. 1996b). Association with the cytoskeletal protein actin (Rosenmund and Westbrook 1993) and neurofilament (Ehlers et al. 1998) occurs through specific binding sites and likely plays a part in intracellular and or membranous distribution of the receptor (Matsuda and Hirai 1999). The role that the

Received September 22, 2000; revised manuscript received November 15, 2000; accepted November 16, 2000.

Address correspondence and reprints requests to L. C. Weaver, Neurodegeneration Research Group, The John P. Robarts Research Institute, PO Box 5015, 100 Perth drive, London, Ontario, N6A 5K8, Canada. E-mail: lcweaver@rri.on.ca

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DRG, dorsal root ganglion; GFP, enhanced green fluorescent protein; HA, hemagglutinin; NR1, NMDA receptor subunit 1; r, recombinant; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
C-terminal exon cassettes play in targeting, clustering and anchoring the NR1 subunits to intracellular or membranous locations has been investigated predominantly in non-neuronal cell lines in culture. Investigations that use organotypic cultures or primary cultures of neuronal cells are required because extrapolation of results obtained in non-neuronal cell lines to specific populations of neurons in vivo may be invalid.

Activation of the NMDA-type glutamate receptor plays an important role in synaptic plasticity (Simon et al. 1992), long-term potentiation (Bliss and Collingridge 1993), central sensitization from a phenomenon called ‘wind-up’ (Liu et al. 1994), excitotoxic injury (Regan and Choi 1991) and contributes to most excitatory neurotransmission events (Krupp and Feltz 1995). The importance of the NMDA receptor in the nervous system and its expression in multiple neuronal phenotypes and glia, make it an attractive target for genetic manipulation. One strategy to manipulate NMDA receptor function is to diminish its presence at the synaptic membrane by saturating the binding sites on specific cytoskeletal elements that facilitate the intracellular trafficking of NMDA receptors. We hypothesize that membrane targeting and synaptic clustering of the endogenous receptor gene may be disrupted by gene transfer of an NMDAR C-terminal construct containing binding sites for neurofilament, α-actinin-2 and actin. Biolistic particle-mediated gene transfer (Arnold et al. 1994; Lo et al. 1994; Thomas et al. 1998) was utilized to induce expression of deletion constructs of the NR1-1a subunit in organotypic cultures of spinal cord slices and dorsal root ganglia (DRG). The expression and subsequent intracellular distribution of the recombinant NR1-1a subunit (rNR1-1) in sensory neurons of organotypic cultured DRG was similar to that reported in cultured cell lines (Matsuda and Hirai 1999; Okabe et al. 1999). We present novel observations of the intracellular distribution of rNR1-1 deletion constructs with, or without, elements of the C-terminus. We report that a rNR1-1 C-terminal construct, consisting of C0, C1 and C2 cassettes, is distributed predominantly to the nucleus, and to a lesser extent to the membrane of sensory neurons. We identify a putative bipartite nuclear localization signal present in the C1 exon cassette and demonstrate that a construct of only the C0 and C2 cassettes is distributed more diffusely throughout the cytoplasm and nucleus. We also present evidence to support the utility of a gene transfer strategy to diminish membranous NR1 content in neurons by coexpressing constructs containing the C1 exon cassette and the full-length rNR1-1.

Materials and methods

Organotypic cultures

Organotypic cultures were made from the spinal cord and DRG of postnatal day 7–9 Wistar rats using the method of Stoppini et al. (1991). Rats were decapitated, the vertebral column and ribs removed and placed in ice-cold slice buffer consisting of Gey’s balanced salt solution (Gibco-BRL) containing 6.5 mg/mL glucose and buffered by 15 mM Hepes (pH 7.2). The spinal cord was removed from the vertebral column in a HEPA-filtered Class A laminar flow hood and placed in a P60 tissue culture dish containing slice buffer. The spinal cord was cut into 400-μm thick slices with a McIlwain tissue chopper contained within a Class B laminar flow hood. The spinal cord slices were floated in a sterile glass Petri dish with slice buffer and transferred to Millicell-CM culture plate inserts (Millipore Corporation, MA, USA). DRG were removed from the vertebral column, placed in slice buffer and transferred onto a Millicell-CM culture plate insert. The Millicell-CM inserts were placed into wells of a six-well plate (Nunc) containing 1.25 mL of medium containing 25% horse serum (Gibco-BRL), 75% minimal essential medium (Gibco-BRL), 15 mM glucose, 15 mM Hepes, 25 μg/mL streptomycin and 25 μg/mL penicillin, pH 7.2. The medium was supplemented with ciliary neurotrophic factor (50 ng/mL) and nerve growth factor (50 ng/mL) for organotypic cultures of spinal cord slices and DRG, respectively. After 24 h, the medium was supplemented with 10 μM of the antimitotics, cytosine arabinoside, 5-fluorodeoxyuridine and uridine (Annis et al. 1990) for 24 h. Subsequently, spinal cord slices and DRG were maintained in medium without antimitotics with a medium change twice weekly.

Plasmids

The NMDAR1 coding region was excised from pHERO (Holmes et al. 2000; Marsh et al. 2000) as a HindIII–AgeI fragment and cloned into the corresponding sites of pcDNA3 such that it was driven by the cytomegalovirus immediate early promoter. Similarly, a HindIII–XbaI fragment was excised from pHERO-NMDAR1 and cloned into corresponding HindIII–XbaI sites in pGFP-N1 such that it was driven by the cytomegalovirus immediate early promoter and fused with enhanced green fluorescent protein (GFP). Recombinant PCR was used to add or remove C-terminal domains and to incorporate restriction endonuclease sites to allow further construction of rNR1-1:GFP fusion proteins, as shown in Fig. 1. Cytomegalovirus-driven GFP in pGFP-N1 was used as a control construct. The rNR1-1:GFP construct tagged at the N-terminal exon cassette with the influenza protein hemagglutinin (HA) was a generous gift of Drs Jerry Lin and Morgan Sheng (Harvard Medical School, Boston).

Particle-mediated gene transfer procedure

Particle-mediated gene transfer was applied to organotypic cultures of spinal cord slices and DRG as described previously (Arnold et al. 1994; Lo et al. 1994; Thomas et al. 1998) with some modifications. The Agracetus PDS-1000/He prototype instrument was replaced with the Bio-Rad Helios Gene Gun. Gold particles (25 mg) 2.6 μm in diameter (Bio-Rad, CA, USA) were weighed and vortexed with 100 μL of 0.05 M spermidine in a 1.5-mL microfuge tube. Plasmid DNA (50 μg) was added in an appropriate volume (<50 μL), vortexed briefly, and 100 μL of 1 M CaCl2 was added dropwise while vortexing. The mixture was kept at room temperature for 10 min and was then centrifuged briefly to pellet the gold particles and the supernatant discarded. The pellet was washed three times with 1 mL of 100% ethanol and the pellet was resuspended in a solution of 0.1 mg/mL polyvinylpyrrolidone in ethanol. This
mixture was applied to the Gold-Coat tubing (Bio-Rad), according to the protocol supplied by the manufacturer. At the time of gene transfer, the six-well plates and culture plate inserts containing spinal cord slices, or DRG, were removed from the incubator and placed in a HEPA-filtered Class A laminar flow hood. The nose of the Helios Gene Gun was placed 1–2 cm directly above the tissue and the gold:DNA particles were discharged into the tissue with 200 psi helium. The organotypic cultures were then maintained for 1–4 days.

Transfection of HEK293 cells with rNR1-1:GFP constructs

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1% L-glutamine and 0.1% penicillin–streptomycin in a 5% CO2 humidified atmosphere. Plasmid DNA (2 μg/well) was prepared with a Qiagen Maxi Kit (Mississauga, Ontario, Canada) and used to transfect HEK293 cells with Lipofectamine (10 μL/well; Life Technologies Inc.) as per the manufacturer’s instructions. Each rNR1-1:GFP construct was cotransfected with a luciferase reporter plasmid (10 ng of pCITE-2 luciferase/well, provided by Dr C. A. Strathdee) to correct for transfection efficiency. Transfected HEK293 cells were harvested and lysed in a commercial cell culture lysis reagent used to assay luciferase activity (Promega, Madison, WI, USA) 48 h after transfection. Protein concentrations for each sample were determined in triplicate using the Bradford assay (Bio-Rad, Mississauga, Ontario, Canada). Total cell protein (10 μg) was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), containing 8 M urea and transferred to nitrocellulose (Amersham, Oakville, Ontario, Canada) using the trans-blot SD semidry transfer cell (Bio-Rad). Immunoblotting was performed with antibodies to GFP (Chemicon, 1 : 200) and to NR1 (Pharmingen, clone 54.1, 1 : 500) using the enhanced chemiluminescence method according to the manufacturer’s instructions (Amersham). Luciferase assays were performed as described previously (Holmes et al. 2000).
Immunohistochemical reactions
Spinal cord slices and DRG were removed from media, washed in phosphate-buffered saline (PBS, pH 7.4), and fixed in 4% formaldehyde in PBS for 1 h at 4°C. Immunoreactivity to the transcription factor NeuN (Chemicon, 1:20 000), to the C2 exon cassette of NR1 (Chemicon, 1:500), and to the α-amino-3-hydroxy-5-methyl-4-isoxale propionic acid (AMPA) receptor GluR2 (Chemicon, 1:500) in the spinal cord slice or DRG was assessed as described previously (Holmes et al. 2000). Briefly, the slices and DRG were incubated for 30 min in Tris–PBS containing 0.3% Triton X-100 detergent (TPBS-X) and then incubated for 2 h in TPBS-X containing 5% normal horse serum (Cedarlane Laboratories). The spinal cord slices and DRG were incubated with the respective primary antibody for 2–3 days in TPBS-X/5% horse serum. Immunoreactivity to the HA epitope tag (Boehringer Mannheim, 1:1500) was assessed similarly, but in the absence of Triton-X. The primary antibody was removed and the tissue was incubated with the respective biotinylated anti-IgG antibody diluted 1:250 for 16–24 h. Immunoreactive neurons were identified by incubating the tissue with rhodamine–streptavidin (Biocan Scientific, Canada) diluted 1:150 for 3 h. All incubations were performed at room temperature.

Fluorescence microscopy
Spinal cord slices and DRG were viewed with a Leitz Diaplan microscope equipped with epifluorescence. Images were captured with a Pro-Series 3CCD digital camera unit. A Zeiss LSM-510 confocal microscope was used to characterize NR1 by indirect immunofluorescence and GFP fluorescence in spinal and sensory neurons. Relative HA immunofluorescence in neurons transfected with HA:NR1-1:GFP alone or in combination with GFP:C0C1C2 or GFP:C0C2 was assessed by obtaining confocal photomicrographs with identical laser settings for each neuron examined. A 5-point scoring system was used to assign a value for the immunofluorescent intensity at the membrane of each transfected neuron. The values for HA:NR1-1:GFP were averaged to generate a mean score. The intensity values for the neurons cotransfected with GFP:C0C1C2, or GFP:C0C2, were then ranked less than, or equal to, the mean score of neurons transfected with HA:NR1-1:GFP alone. Photomicrographs were processed using IMAGE PRO PLUS software (Media Cybernetics, LP), LSM 510 software (Zeiss), ADOBE PHOTOSHOP 5.02 (Adobe Systems Inc.) and COREL DRAW 8 (Corel Inc.).

Results
Spinal cord slice and DRG morphology in culture
The antibody to the neuron-specific transcription factor, NeuN, revealed immunoreactive neuronal nuclei throughout the gray matter, but not the white matter of the spinal cord slice (Fig. 2a). Higher magnification of NeuN immunoreactivity in spinal cord slices illustrated oval nuclei, located centrally within large, multipolar neurons in the ventral horn (Fig. 2b). Similarly, a cluster of NeuN immunoreactive neurons was obvious within the DRG (Fig. 2c) and increased magnification demonstrated numerous round sensory neurons with oval nuclei (Fig. 2d).

Biologic particle-mediated gene transfer
Green fluorescent cells were abundant in the spinal cord slice and DRG after particle-mediated gene transfer of rNR1-1:GFP. In the spinal cord slice, the fluorescent cells were distributed throughout the white matter and were negligible in the gray matter (Fig. 2e). These cells were not neuronal because NeuN-immunoreactive cells did not

Fig. 2 Photomicrographs demonstrating neurons within the organotypic cultures of the spinal cord slice and DRG. (a) Immunoreactivity to the neuron-specific transcription factor NeuN revealed nuclei of neurons located in the gray matter (GM) and not the white matter (WM) of a spinal cord slice after 5 days in culture. (b) Increased magnification of cells in the ventral horn with confocal microscopy demonstrates intact neurons with intensely labeled nuclei. Similarly, in (c), NeuN-immunoreactive neurons are observed in a cluster in the ganglion (G). (d) With increased magnification and confocal microscopy of the DRG, many neurons with intact oval nuclei were observed. The extent and location of gene transfer to cells is depicted in photomicrographs of the spinal cord slice (e) and the dorsal root ganglion (f). In (e) all GFP fluorescent, transfected cells are located in the white matter of the spinal cord slice and are not neurons. In (f) GFP fluorescent, transfected neurons are located in the ganglion (G) and non-neuronal, GFP fluorescent cells are also present in the dorsal root (DR). Calibration bar is 250 μm in (a, c, e and f); 20 μm in (b and d).
demonstrate GFP fluorescence. Particle-mediated gene transfer into neurons in the gray matter of the spinal cord slice was unsuccessful, possibly because of inadequate penetration of the biolistic particles. The particles penetrated 10 μm at 200 psi helium. Increasing the helium pressure was attempted to improve penetration, but the spinal cord slices were dislodged from the filter insert. Penetration of biolistic particles into the DRG was adequate because numerous rNR1-1:GFP fluorescent neuronal and non-neuronal cells were observed in the DRG (Fig. 2f). An average of 12 ± 3 sensory neurons per ganglion (n = 20) expressed GFP 3 days after particle-mediated gene transfer.

**Distribution of NMDAR constructs**

Expression of the rNR1-1:GFP fusion protein constructs described in Fig. 1 each demonstrated a unique distribution of fluorescence within sensory neurons distinct from the GFP control construct (Fig. 3). In each sensory neuron, rNR1-1:GFP expression was excluded from the nucleus and was present at the membrane, localized in discrete subcellular domains (Fig. 3a), and in punctate fashion in the process (Fig. 3b). When the C-terminus was removed to form rN:GFP, the expression pattern was altered. rN:GFP was expressed diffusely throughout the cytoplasm and was excluded from the nucleus (Fig. 3c). Membrane localization or punctate expression in neuronal processes did not occur. The GFP fusion construct containing only the C-terminus, GFP:C0C1C2, was localized predominantly in the nucleus of sensory neurons with only minor distribution to the membrane and neuronal processes (Fig. 3d). The C-terminal construct lacking the C1 cassette, GFP:C0C2, was also localized to the nucleus of sensory neurons, but less intensely than the nuclear localization observed for GFP:C0C1C2. In contrast to GFP:C0C1C2, the GFP:C0C2 construct demonstrated substantial cytoplasmic distribution and no obvious membranous expression (Fig. 3e). These expression patterns contrasted sharply with the diffuse cytoplasmic and nuclear fluorescence in cells after gene

---

**Fig. 3** Confocal micrographs demonstrating distribution patterns of the rNR1-1 constructs in sensory neurons three days after biolistic particle-mediated gene transfer. (a) The expression of rNR1-1:GFP construct in a sensory neuron is localized in the cytoplasm, in membranous clusters and is excluded from the nucleus. (b) A three-dimensional projection of 11 images, 1.5 μm apart, of a neuronal process emanating from a sensory neuron illustrates that the expression of rNR1-1:GFP also extends into the process in which the distribution is punctate (dotted arrow). Because of greater fluorescent intensity of the soma relative to the process, the neuron soma shown was captured in a separate confocal micrograph and then merged with the micrograph of the process. (c) Expression of the rN:GFP construct is present only in the cytoplasm. (d) Expression of the GFP:C0C1C2 construct is localized predominantly in the nucleus. (e) The GFP:C0C2 construct is distributed to the nucleus and the cytoplasm. (f) Expression of the GFP control construct was diffuse throughout the cytoplasm and nucleus. A solid arrow indicates the nucleus of each cell. Calibration bars are 10 μm. (g) Immunoblot analysis of rNR1-1:GFP chimera expression in HEK293 cells using an anti-NR1 antibody and an anti-GFP antibody 48 h post transfection with (h) the corresponding densitometric analysis. Data represent optical densities (OD)/μg of protein/relative luciferase unit (RLU).
transfer of the GFP control construct (Fig. 3f). The non-neuronal cells in the spinal cord slice also expressed the rNR1-1:GFP constructs after particle-mediated gene transfer. These cells demonstrated patterns of expression for each of the constructs that were similar to those observed in the sensory neurons (data not shown).

The relative level of expression of each of the GFP fusion proteins was determined after transient transfection of HEK293 cells in culture. Antibodies against NR1 or GFP in a western blot of cell homogenates demonstrated similar levels of fusion protein expression (Fig. 3f). Densitometric analysis of the western blot of transfected cell homogenates

Fig. 4 The distribution and expression of the AMPA receptor GluR2 is similar in transfected and nontransfected sensory neurons. (a) GluR2 immunoreactivity in numerous sensory neurons in a DRG demonstrates the endogenous expression level in small and large diameter neurons. (b) A sensory neuron transfected with rNR1-1:GFP and in (c) exhibiting normal GluR2 distribution. (d) A sensory neuron transfected with GFP:C0C2 and in (e) exhibiting similar GluR2 expression as neighboring neurons. (f) A sensory neuron cotransfected with rNR1:GFP + GFP:C0C1C2 exhibiting characteristic GFP fluorescence, as well as (g) typical GluR2 immunoreactivity. Calibration bar in (a) 20 μm, in all others, 10 μm.

Fig. 6 The membranous distribution of rNR1-1 is confirmed by immunoreactivity to an N-terminal HA epitope tag on rNR1-1. (a) The distribution of the fusion protein, HA:rNR1-1:GFP, is predominantly cytoplasmic with membranous patches as demonstrated by GFP fluorescence. (b) Membranous expression of HA:rNR1-1:GFP is confirmed by immunoreactivity to HA in nonpermeabilized cells. (c) After cogene transfer of a sensory neuron with HA:rNR1-1:GFP and GFP:C0C1C2, GFP fluorescence is intense in the nucleus, but decreased at the membrane. (d) Immunoreactivity to HA provides evidence to support reduced membranous expression of HA:rNR1-1:GFP after cogene transfer with GFP:C0C1C2. (e) GFP fluorescence is observed throughout the neuron, including nuclear and membranous expression, after cogene transfer with HA:rNR1-1:GFP and GFP:C0C2. (f) Immunoreactivity to HA, confirms that expression of HA:rNR1-1:GFP is still abundant on the membrane and has not been substantially altered by cogene transfer with GFP:C0C2. A solid arrow indicates the location of the nucleus. The intensity of the ‘red’ laser was increased by 20% to obtain the photomicrograph in (d) and was similar when obtaining the micrographs in (b) and (f). Calibration bars 10 μm.
show that the expression levels of the GFP fusion proteins are within 2-fold of each other (Fig. 3g).

**GluR2 expression is unaltered in rNR1-1-transfected neurons**

The AMPA receptor GluR2 was observed in numerous sensory neurons in the cultured DRG (Fig. 4a). After particle-mediated gene transfer of rNR1-1:GFP (Figs 4b and c), or the C-terminal construct GFP:C0C2 (Figs 4d and e), the expression pattern of GluR2 was unchanged. Similar results were obtained for the combined gene transfer of HA:rNR1:GFP plus GFP:C0C1C2 (Figs 4f and g). This suggests that membranous distribution and trafficking of AMPA receptors remains unaltered after biolistic particle-mediated gene transfer and over-expression of rNR1-1 and the C-terminal constructs in sensory neurons.

**Endogenous NR1 and rNR1-1 expression levels after gene transfer**

An antibody to the C2 cassette of NR1 was used to detect endogenous NR1 as well as the full length and C-terminal rNR1-1 deletion constructs. The level of endogenous NR1 expression in sensory neurons was much less than the expression levels generated by biolistic gene transfer of rNR1-1:GFP (Fig. 5a). Neuronal processes were observed extending from sensory neurons and coursing from the ganglion after gene transfer with GFP:C0C1C2 (Fig. 5b). At high magnification, the endogenous pattern of NR1 expression (Fig. 5c) was observed to be cytosolic with membranous patches. Endogenous NR1 immunoreactivity in processes extending from sensory neurons was not discernible above background fluorescence. The pattern of rNR1-1:GFP expression in sensory neurons was indistinguishable from that of the endogenous NR1 immunoreactivity (Fig. 5d).

**The importance of the C1 exon cassette for membranous distribution of rNR1-1**

The C-terminus of NR1-1 is the longest of the NR1 splice variants and consists of C1 and C2 exon cassettes in addition to the C0 cassette that is common to all NR1 splice variants. The C1 cassette is found only in NR1-1 and NR1-3 splice variants and has many important features integral to receptor function that have been characterized (Tingley et al. 1993; Ehlers et al. 1996b; Tingley et al. 1997). Despite the limited distribution of the GFP:C0C1C2 construct to the membrane, we tested our hypothesis that over-expression of a C-terminal construct containing C1 would disrupt localization of rNR1-1 at the membrane. The intense rNR1-1 expression in the cell soma and neuronal processes after gene transfer, in comparison to the low levels of endogenous NR1 expression, obscured visualization of the endogenous receptor (see Figs 4a and b). This made it impossible to assess the impact of C-terminus over-expression on the distribution of endogenous gene expression in sensory neurons. To circumvent this problem, a cogene transfer strategy was used in which biolistic particles were coated with a 1:1 mixture of GFP:C0C1C2 and HA:rNR1-1:GFP, or GFP:C0C2 and HA:rNR1-1:GFP, prior to gene transfer into DRG neurons. The HA epitope tag in the N-terminus exon cassette made a direct immunohistochemical assessment of membranous rNR1-1 expression possible in nonpermeabilized sensory neurons. The impact on membranous distribution of HA:rNR1-1 by GFP:C0C1C2 was then compared with that of GFP:C0C2 that does not contain the C1 exon cassette.

Gene transfer of HA:rNR1-1:GFP into a sensory neuron and visualized by GFP fluorescence is demonstrated in Fig. 6(a). Note the concentration of fluorescence on the perimeter of the cell and within the cytoplasm and the lack of fluorescence in the nucleus. The corresponding immunoreactivity to HA, in the same neuron, is portrayed in Fig. 6(b). The HA immunofluorescence is clearly on the membrane of the nonpermeabilized cell and is not cytosolic.
like the GFP fluorescence in Fig. 6(a). After combined gene transfer of HA:rNR1-1:GFP plus GFP:C0C1C2, the GFP fluorescence remained cytosolic, but was also intense within the nucleus (Fig. 6c) due to the presence of GFP:C0C1C2, as observed previously when GFP:C0C1C2 was transferred alone (Fig. 3d). Immunoreactivity to HA was apparent on the membrane (Fig. 6b) and the relative intensity of the fluorescence was similar to that of HA:rNR1-1:GFP alone (Fig. 6b) and was greater than that seen after combined gene transfer of HA:rNR1:GFP and GFP:C0C1C2 (Fig. 6d). These results were repeated in 10 sensory neurons in 7–9 separate DRG after combined gene transfer with HA:rNR1-1:GFP plus GFP:C0C2. Of these 10 sensory neurons, only 3 had diminished fluorescent intensity of HA immunoreactivity compared with gene transfer with HA:rNR1-1:GFP alone.

Nuclear localization of the C1 cassette-containing construct

We examined the amino acid sequence of the C-terminus from NR1-1 to explore possible causes for the concentration of the GFP:C0C1C2 construct in the nucleus of sensory neurons. We discovered a putative bipartite nuclear localization signal (NLS) within the peptide sequence, KKKAT-FRAITSTLASSFKRRR, in the C1 exon cassette that is similar to bipartite NLS sequences reported previously (Dingwall and Laskey 1991). To confirm the nuclear localization of the construct we compared the fluorescence of GFP:C0C1C2 (Fig. 7a) with immunoreactivity to the nuclear transcription factor NeuN (Fig. 7b). We found that GFP:C0C1C2 fluorescence colocalized with immunoreactivity to NeuN in the nucleus of sensory neurons. In comparison, particle-mediated gene transfer of neurons with the GFP:C0C2 construct that lacks the C1 exon cassette, exhibited diffuse fluorescence throughout the cytoplasm and nucleus (Fig. 7c) and resembled that of the GFP control construct (Fig. 3e). The fluorescent intensity of the nucleus after particle-mediated gene transfer of neurons with GFP:C0C2 (Fig. 7c) was significantly less than that of the nucleus of neurons gene transferred with GFP:C0C1C2 (Fig. 7a). This suggests that the GFP:C0C2 enters the nucleus by passive diffusion, similar to GFP alone, whereas the GFP:C0C1C2 is localized to the nucleus by an active process.

The nuclei of sensory neurons were easily observed in the DRG by their immunoreactivity to NeuN. Particle-mediated gene transfer did not affect the nucleus of sensory neurons because an intact, oval nucleus was observed in cells expressing the GFP control construct, or in cells expressing the other rNR1-1:GFP constructs. However, fragmented nuclei were frequently observed in neurons following gene transfer with the GFP:C0C1C2 construct (Fig. 7d). Particle-mediated gene transfer of the GFP:C0C1C2 construct resulted in 40% of these neurons exhibiting nuclear fragmentation. In contrast, intact oval nuclei, without fragmentation, were observed in sensory neurons after gene transfer of GFP:C0C2 (Fig. 7c).
Discussion

The NMDA receptor is a common pharmacological target for the alleviation of chronic pain (Hao and Xu 1996), muscle spasticity (Schwarz et al. 1992) or epilepsy resulting from trauma or neurodegenerative diseases (reviewed in Parsons et al. 1999). In sensory neurons, the NMDAR plays an important role in the sensory perception of pain by mediating the 'wind-up' phenomenon and central sensitization (Liu et al. 1994). We demonstrate proof of principle that the NMDA receptor may also be a suitable target for molecular therapy. Chronic attenuation of signaling via the NMDA receptor would have numerous positive outcomes after traumatic injuries, such as spinal cord injury, that elicit chronic pain states, autonomic dysfunction and/or spasticity.

The intensity of NMDA-induced signaling depends on the quantity of neurotransmitter released from the presynaptic terminal and the density of NMDAR at the postsynaptic membrane. An intracellular mechanism, likely acting through specific motifs present in the C-terminus of the NR1 and NR2 subunits, directs the subunits to intracellular or synaptic locations (Chazot and Stephenson 1997; Okabe et al. 1999). Expressed at sufficient levels, C-terminal constructs of rNR1-1 may interact with cytoskeletal proteins and disrupt the proper interaction of the native NMDAR to these proteins that facilitate receptor clustering at the membrane, thereby diminishing NMDA-induced signaling (Dong et al. 1997; Matsuda and Hirai 1999). Gene transfer of numerous sensory neurons with recombinant NR1 constructs by biolistic particle-mediated gene transfer provided an opportunity to investigate disruption of receptor clustering at the membrane of neurons within organotypic neural tissue. The clustered, membranous distribution of the NR1-1 subunit that we observed in sensory neurons after biolistic particle-mediated gene transfer was similar to that observed previously in primary cultures of neurons and in fibroblast cell lines (Ehlers et al. 1995; Matsuda and Hirai 1999; Okabe et al. 1999). The diffuse cytoplasmic expression and lack of membranous clusters that result from gene transfer of rN:GFP in sensory neurons supports the documented importance of the C-terminus for membrane localization (Ehlers et al. 1995). The absence of NR1-1 membranous clustering after disruption of actin filaments has demonstrated the importance of the cytoskeleton in this process (Allison et al. 1998; Matsuda and Hirai 1999). The C0 cassette contains an interaction site for α-actinin-2 (Wyszenski et al. 1997) that may mediate a linkage with actin filaments. Likewise, disruption of microtubules (Matsuda and Hirai 1999) prevents formation of NR1-1 membranous clusters normally caused by interaction of the C1 exon cassette with binding sites for neurofilament (Ehlers et al. 1998). When the C0, or C1, exon cassettes are over-expressed in fibroblast cells that lack NR2, expression of transfected NR1-1 is prevented from forming membranous clusters (Matsuda and Hirai 1999). In our study, the HA epitope tag in the N-terminus HA:rNR1-1:GFP construct, permitted a direct assessment of membranous rNR1-1 expression. Our observations after HA:rNR1-1:GFP gene transfer alone, and after the combined gene transfer with the C-terminal constructs, GFP:C0C1C2 or GFP:C0C2, suggest that the presence of the C1 exon cassette in the C-terminal construct enhances the disruption of membranous expression of rNR1-1 in DRG sensory neurons. Therefore, the findings in fibroblasts pertaining to the C1 exon cassette remain valid for sensory neurons in organotypic culture. In contrast, we found little evidence to suggest that the C0 and C2 cassettes, present in GFP:C0C2, were as important as the C1 cassette in disrupting membranous distribution of rNR1-1.

A novel observation of this investigation is the concentration of the C-terminus construct GFP:C0C1C2 in the nucleus of sensory neurons. The concentration of the GFP:C0C1C2 construct in the nucleus can be explained by the presence of a putative NLS in the C1 exon cassette. Typical of a bipartite NLS, the sequence is rich in arginine and lysine and consists of two runs of basic amino acids separated by a spacer region (Dingwall and Laskey 1991). In our preliminary studies, the removal of the C1 exon cassette from the C-terminus led to diffuse expression of GFP:C0C2 throughout the cytoplasm and nucleus, demonstrating passive diffusion rather than concentration in the nucleus by an active mechanism (K. D. Holmes et al. unpublished). The relevance of the NLS in the C1 exon cassette of the NR1-1 is unknown. C-terminal domains that contain a NLS have been reported to undergo cleavage from membrane-bound receptors (Struhl and Adachi 1998; Bao et al. 1999; Brown et al. 2000). Once inside the nucleus, these domains act directly, or indirectly, as transcription factors (Struhl and Adachi 1998; Brown et al. 2000). A potential link between the activation of nuclear transcription factors, nuclear factor kappa β and p53, glutamatergic stimulation and apoptosis has already been reported (Grilli and Memo 1999; Uberti et al. 2000). The nuclear fragmentation that we observed was not an artifact of biolistic particle-mediated gene transfer or over-expression of rNR1-1, because fragmented nuclei were only observed after gene transfer of the GFP:C0C1C2 construct. Therefore, the nuclear fragmentation was a specific outcome, either directly or indirectly, of the presence of this construct in the nucleus.

The NR1 splice variants are differentially expressed throughout the PNS and CNS (Nakanishi et al. 1992; Ishii et al. 1993; Petralia et al. 1994; Tolle et al. 1995). A strategy to disrupt membrane distribution based on the C1 exon cassette would have limitations and advantages due to this tissue-specific expression of splice variants. In tissues in which expression of NR1-1 is abundant, such as brain and DRG, the strategy would be effective. In other tissues, such
as spinal cord, in which NR1 splice variants, NR1-4 and NR1-2, are predominant, the strategy may not be as effective. With appropriate gene transfer, this selective expression may permit targeting specific populations of neurons and sparing others. A rNR1-1 construct containing the C1 exon cassette may not be ideal for gene transfer because of the presence of the putative NLS and prevalent nuclear fragmentation in sensory neurons. An alternative C1 exon-containing construct with a mutated NLS is currently being tested in our laboratory. C-terminal constructs derived from the other NR1 splice variants and from NR2 subunits are also being investigated. These constructs may also prove to have more utility in disrupting the clustering and/or anchoring of the NMDA receptor complex at the membrane.

Successful manipulation of the NMDA receptor by gene transfer vectors must accomplish not only gene induction, but also assembly of the transgene into the receptor complex and distribution to the synaptic membrane where it can compete with, or displace, native receptors. Our study demonstrated the use of biolistic particle-mediated gene transfer to deliver a physiologically relevant gene to organotypic neural tissue. Not only was gene transfer successful, but intracellular distribution of the rNR1-1:GFP fusion protein was similar to that of the native receptor protein. Our results with the NR1-1 gene have lent credence to our strategy of inducing expression of a recombinant NMDA receptor subunit that can be assimilated into the NMDA receptor complex. Furthermore, we demonstrated that an NR1-1 C-terminal construct diminishes membranous expression of rNR1-1 in sensory neurons, possibly by interfering with cytoskeletal elements that normally anchor the receptor complex to the membrane.

Acknowledgements

This research was supported by a grant from the Medical Research Council of Canada and from the Ontario Neurotrauma Foundation. LCW is a recipient of a Career Investigator Award from the Heart and Stroke Foundation of Ontario. The Ontario Neurotrauma Foundation supports DRM with a fellowship award and KDH with a studentship award.

References


© 2001 International Society for Neurochemistry, Journal of Neurochemistry, 77, 23–33


Parsons C. G., Danyus W. and Quack G. (1999) Memantine is a clinically well tolerated \( N \)-methyl-\( \alpha \)-aspartate (NMDA) receptor antagonist – a review of preclinical data. *Neuropharmacology* **38**, 735–767.


