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N-Methyl-D-Aspartate receptor subunit phenotypes of vagal afferent neurons in nodose ganglia of the rat

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Abstract

Most vagal afferent neurons in rat nodose ganglia express mRNA coding for the NR1 subunit of the heteromeric NMDA receptor ion channel. NMDA receptor subunit immunoreactivity has been detected on axon terminals of vagal afferents in the dorsal hindbrain, suggesting a role for presynaptic NMDA receptors in viscerosensory function. Although NMDA receptor subunits (NR1, NR2B, NR2C, and NR2D) have been linked to distinct neuronal populations in the brain, the NMDA receptor subunit phenotype of vagal afferent neurons has not been determined. Therefore, we examined NMDA receptor subunit (NR1, NR2B, NR2C, and NR2D) immunoreactivity in vagal afferent neurons. We found that, although the left nodose contained significantly more neurons (7603), than the right (5978), the proportions of NMDA subunits expressed in the left and right nodose ganglia were not significantly different. Immunoreactivity for NMDA NR1 subunit was present in 92.3 % of all nodose neurons. NR2B immunoreactivity was present in 56.7% of neurons, NR2C expressing nodose neurons comprised 49.4% of the total population, and NR2D subunit immunoreactivity was observed in just 13.5% of all nodose neurons. Double labeling revealed that 30.2% of nodose neurons expressed immunoreactivity to both NR2B and NR2C, while NR2B and NR2D immunoreactivity were co-localized in 11.5% of nodose neurons. NR2C immunoreactivity co-localized with NR2D in 13.1% of nodose neurons. Our results indicate that the majority of vagal afferent neurons express NMDA receptor ion channels composed of NR1, NR2B and NR2C subunits, and that a minority phenotype that expresses NR2D also expresses NR1, NR2B, and NR2C.

Keywords

NMDA receptors; vagal afferent neurons; nodose ganglion

Introduction

Multiple lines of evidence suggest that glutamate is a fast excitatory transmitter released by the central terminals of primary vagal afferent neurons (Andresen and Mendelowitz, 1996). For example, vagal afferent neurons contain vesicular glutamate (Lawrence, 1995; Saha et al., 1995; Brouns et al., 2004). These neurons also express phosphate-activated glutaminase (Li et al., 1996; Zhuo et al., 1997), a hydrolytic enzyme that converts glutamine to glutamate, and is an indicator for glutamatergic neurotransmission (Chiba and Kaneko,

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1993). Stimulation of the vagus nerve or solitary tract evokes glutamate release in the NTS (Granata et al., 1984; Allchin et al., 1994), and the stimulation-induced release of endogenous glutamate in the NTS also is evoked by physiological activation of vagally innervated structures such as baroreceptors (Lawrence, 1995). Glutamatergic vagal afferent synapses have been shown to activate dissociated NTS neurons in primary culture (Jin, Y. H., et al 2004). Finally, pharmacological experiments indicate that vagal afferent neurotransmission and functional responses evoked by vagal afferent stimulation are antagonized by both NMDA-type and non-NMDA-type glutamate receptor antagonists applied to the area of central vagal afferent termination in the NTS (Machado et al., 1997; Seagard et al., 2001; Milner and Pickel, 2003).

Although NMDA receptors often are associated with excitatory postsynaptic effects, there also is evidence that NMDA receptors function pre-synaptically in glutamatergic transmission. NMDA receptor subunit immunoreactivity has been localized to axon terminals at a variety of sites in the brain (Aicher et al., 1999; Paquet and Smith, 2000; Casado et al., 2000). Furthermore, electrophysiological data indicates that presynaptic NMDA receptors modulate transmitter release and thereby the frequency of mini excitatory postsynaptic currents (EPSCs) in the visual cortex (Sjostrom et al., 2003). Similar results have been obtained in entorhinal cortex, where putative presynaptic NMDA receptors increase the frequency of mini EPSCs (Berretta and Jones, 1996). Finally recent results strongly implicate NMDA receptors on spinal primary afferent terminals in modulating release of glutamate from somatosensory afferents (Bardoni et al., 2004).

NMDA receptor ion channels are heteromeric assemblies composed of the subunit, NR1, in combination with NR2A, B, C and/or D, and/or NR3A subunits to form an NMDA ion channel (Goebel and Poosch, 1999; Cull-Candy et al., 2001). Not all NMDA receptors contain all of the NR2 subunits. Rather, various neuronal populations express distinct NMDA receptor subunit phenotypes, which may be related to differences in NMDA receptor function (Akazawa et al., 1994; Boyce et al., 1999; Dingledine et al., 1999; Cull-Candy et al., 2001; Clayton and Browning, 2001; Cull-Candy and Leszkiewicz, 2004).

Information on NMDA receptor subunit expression in the peripheral nervous system is limited, but expression of one or more subunits of the NMDA receptor has been reported in enteric, somatosensory and viscerosensory neurons (Shigemoto et al., 1992; Burns et al., 1994; Marvizon et al., 2002; Chang et al., 2003). Of particular interest is the fact that NMDA NR1 message has been detected in a majority of vagal afferent neurons of the nodose ganglia (Shigemoto et al., 1992; Chang et al., 2003), and NMDA receptor subunit immunoreactivity has been demonstrated in vagal afferent terminals in the hindbrain (Benke et al., 1995; Andres et al., 1996; Aicher et al., 1999; Huang and Pickel, 2002, Huang and Pickel, 2003). These anatomical data support a role for presynaptic NMDA receptor function in neurotransmission by primary vagal afferent neurons. Nevertheless, we remain largely ignorant of the variety of NMDA receptor subunit phenotypes expressed by vagal afferent neurons. Because the subunit composition of NMDA receptors expressed by vagal afferents may be important for understanding the nature of glutamate participation in visceral afferent neurotransmission, we used immunocytochemistry to reveal the subunit composition of NMDA receptors expressed by primary vagal afferent neurons of nodose ganglia in the rat.

Materials and Methods

Animals

Male Sprague-Dawley rats (6 weeks old, Simonsen Labs, Gilroy, CA) were individually housed in a temperature-controlled vivarium with ad libitum access to a standard pelleted rat

chow (Harlan Teklad F6 Rodent Diet W, Madison, WI) and water. The rats were maintained on a 12:12-h light-dark schedule and were habituated to the laboratory conditions for 3 to 5 days prior to collection of tissues for immunocytochemistry. All animal procedures were approved by the Washington State University Institutional Animal Care and Use Committee and conform to National Institutes of Health guidelines for the use of vertebrate animals (publication No. 86–23, revised 1985).

Immunofluorescence and Counter-staining

Rats (n=8) were deeply anesthetized with an intramuscular injection of acepromazine, ketamine, and xylazine (0.1ml/100gm), exsanguinated and transcardially perfused, using a sequence of phosphate buffered saline, (PBS) and 4% paraformaldehyde solution (pH 7.4). Immediately following the perfusion, nodose ganglia were collected, post-fixed (4% paraformaldehyde, 30 min), and immersed overnight in a cryoprotectant solution of 18% sucrose in PBS and NaN_3 . The ganglia were cut longitudinally into 20- μm serial sections, mounted in four series on glass slides (Superfrost Plus), with every fourth section on the same slide. Generally this procedure yielded 28–32 20 μm thick sections per ganglion, with each slide containing 7–8 20 micron sections with an interval of 60 microns between adjacent sections. After drying on the slides, the sections were rehydrated and processed for detection of selected NMDA receptor subunits according to conventional immunofluorescence protocols. Briefly, the slide-mounted sections were immersed for 15 min in a 0.1% sodium borohydride solution to reduce autofluorescence (Clancy and Cauller, 1998). Subsequently, the sections were incubated overnight in a blocking solution consisting of 10% normal horse serum in Tris sodium phosphate buffer (TPBS, pH 7.4). The blocking solution was washed from the tissues, and each section was incubated for 48h, at room temperature in affinity-purified polyclonal primary antisera (Table 1.) raised in goat (Santa Cruz Biotechnology, Santa Cruz, CA) against the NR1 (sc-1467, 1:100), NR2B (sc-1469, 1:250), NR2C (sc-1470, 1:500), or NR2D (sc-1471, 1:500) NMDA receptor subunit. These antibodies had been previously tested for their ability to detect NMDAR subunits (Becker et al., 1998; Li et al., 1998; Marvizon et al., 2002). Western blots of yeast extracts revealed no cross-reactions between the antibodies against the NR1 and NR2 subunits and only weak cross-reactions among the NR2 subunit antibodies. Alternatively, for some dual labeling experiments, primary antisera raised in rabbit against NR2B (Zymed 71-8600, 1:100) and NR2C (Abcam ab110, 1:200) subunits were substituted for comparable, goat-derived, primary antisera. Although the rabbit NR2C antisera has been reported to detect NR2B in western blots from the cerebellum of young rats (Monyer et al., 1994; Kennedy, 1997), we found that, when nodose tissue was double stained using goat anti-NR2C and rabbit anti-NR2C, there was 100% coincidence in staining by these antisera, with no additional staining. Since the goat anti-NR2C does not recognize the NR2B subunit, we believe that the rabbit anti-NR2C is specific for NR2C in the nodose of adult rats. Four rats were used for single labeling, and four rats were used for double labeling of nodose neurons expressing NMDA receptor subunits. In double labeling experiments we used the following mixtures of primary antisera (NR2B rabbit/NR2C goat, NR2B rabbit/NR2D goat and NR2C rabbit/NR2D goat). After three washes in TPBS, sections were incubated overnight at room temperature in an appropriate secondary antibody raised in donkey (CY3, 1:200, 705-165-003, Jackson ImmunoResearch, West Grove, PA) for single staining or in an appropriate mixture of secondary antisera raised in donkey (CY3) and goat (Alexa Fluor 488, 1:400; Molecular Probes A21206) for double staining. After the overnight incubation, the sections were washed with tris buffered PBS, counter-stained with DAPI (Molecular Probes, Eugene, OR), to reveal neuronal and glial nuclei, and mounted in ProLong (Molecular Probes, Eugene, OR) to reduce photo-bleaching.

To control for specificity of the primary antibodies, separate sets of sections were incubated without primary antisera and others were pre-incubated with the immune serum plus the peptide antigen used to raise the antisera prior to application to the tissue. Both of these control procedures eliminated immunofluorescent staining in the nodose for all of the antisera we used.

Counting of nodose ganglion neurons

Images of the nodose ganglia were viewed and captured using a Zeiss Axioplan 2 imaging photomicroscope equipped with a digital camera (Axio Cam MRc) and appropriate filters for DAPI, CY3 and Alexafluor 488. With the aid of a computer, the captured images were evaluated with the Axio Vision 4.0 Imaging system (Carl Zeiss Vision, Oberkochen, Germany). For preparation of microscopic illustrations, Corel Graphic Suite 11 was used to enhance contrast and sharpness of the digital images, to add lettering, and to make composite plates.

In order to determine the total number of neurons in the nodose ganglia, DAPI-stained neuronal nuclear profiles were counted under $\times 200$ magnification in all of the sections for all series from a given ganglion. Neuronal nuclear profiles were easily discriminated from glial nuclei by virtue of their large size, and spherical to slightly ovoid shape. All neuronal nuclei were counted regardless of the NMDA receptor subunit phenotype with which they were associated. In order to avoid double counting, diameters of all nuclear profiles in eight random sections from both the left and the right nodose ganglia were measured using the Axio Vision 4.0 Imaging system (Carl Zeiss Vision, Oberkochen, Germany). The combination of serial counts and average nuclear diameters were used to estimate the total number of neurons in each ganglion as the average sum of nuclear profiles per ganglion corrected by Abercrombie's equation (Ebbesson and Tang, 1965). Only neurons that exhibited a clear nuclear shadow were counted. The rationale for using every fourth section was to eliminate the likelihood of counting the same neuron twice in these 20- μm sections, since the long axis of neurons located in nodose ganglia has been reported to be 15–35 μm (Carobi, 1996).

Differences in cell number or expression of NMDA receptor subunit immunoreactivity were tested for statistical significance using Student's t-test ($P \leq 0.05$). Data are expressed as means $\pm$ SEM.

Results

Nuclear diameters and neuron counts

Nuclear profiles in DAPI-stained nodose ganglion neurons were round. The average diameter of these profiles in left and right nodose ganglia was 13.4 μm and 12.9 μm , respectively. The maximum diameter of a nuclear profile in a left nodose ganglion was 18.9 μm , while 18.7 μm was the largest nuclear diameter in a right nodose ganglion. The minimum diameter of the smallest visible fragment of a nuclear profile was measured in the left (6 μm) and right (7.5 μm) nodose ganglion as well.

The combination of average and maximum nuclear diameters, size of the smallest visible fragments, and section thicknesses were used to calculate the corrected number of neurons per ganglion, using a previously described correction formula (Ebbesson and Tang, 1965). The corrected average numbers of neurons in the left and right nodose ganglia respectively were 7603 ± 185 and 5978 ± 432 .

Expression of NMDA receptor subunits in the nodose ganglia

Each section of nodose ganglion stained with antisera against one of the NMDA receptor subunits (NR1, NR2B, NR2C, and NR2D) contained positively labeled neurons (Fig. 1A–D). No significant differences in the proportion of neurons expressing immunoreactivity for the various subunits combinations were observed between the left and right nodose ganglia and, therefore, the following results represent pooled data for both right and left sides.

Analysis of single labeling revealed that $92.3\% \pm 1.5\%$ of nodose ganglion neurons expressed NR1-immunoreactivity (–IR) (Fig. 2). The bars in Fig. 2 represent the percentage of neurons that express a particular phenotype out of the total number of nodose neurons $\pm$ SEM. NR1-IR was found in perikarya of neurons of all sizes throughout the nodose ganglia. Many of the NR1-IR positive neurons were situated along the margins of the nodose, although central portions of the ganglia were populated with immunoreactive cells as well (Fig. 3A).

NR2B-IR was observed in $56.7\% \pm 3.0\%$ of nodose ganglion neurons (Fig. 2). Clusters of large to medium-sized NR2B-IR neurons were commonly seen in cranial and marginal regions of nodose ganglia. However, individual medium-sized profiles could also be found in other regions of the ganglia. No differences in NR2B staining intensity were seen within the nodose ganglion neurons.

NR2C-immunoreactivity was present in $49.4\% \pm 1.9\%$ of nodose ganglion neurons, while just $13.5\% \pm 2.0\%$ were NR2D-IR (Fig. 2). NR2C-IR or NR2D-IR neurons were primarily small and medium-sized and were randomly distributed throughout the nodose ganglia (Fig. 3B,C). NR2C-IR perikarya were very intensely stained and formed clusters of cells (Fig. 3C), whereas NR2D-IR profiles were less intensely stained and presented primarily as scattered single neurons with relatively few neuronal clusters (Fig. 3B).

Double labeling immunofluorescence revealed co-localization of NR2 subunits within the nodose ganglion neurons (Fig. 4A, B). In the population of nodose ganglion neurons $30.2\% \pm 1.8\%$ co-expressed NR2B and NR2C immunoreactivity (Fig. 2). NR2B/NR2D and NR2C/NR2D immunoreactivity was found in $11.5\% \pm 1.7\%$ and $13.1\% \pm 1.9\%$ of nodose ganglion neurons respectively (Fig. 2).

Discussion

There are only a few previously published estimates of the number of neurons in the rat nodose ganglia (Cooper, 1984; Bakal and Wright, 1993; Carobi, 1996; Chang et al., 2003; Wei et al., 2005). Our estimates for the total number of neurons in the nodose ganglia are in general agreement with most other investigators, who report numbers in the range of 5000 to 7000 neurons per ganglion (Cooper, 1984; Carobi, 1996; Chang et al., 2003; Wei et al., 2005). Of published estimates, only Carobi, et al. (Carobi, 1996) noted a significant difference in neuronal numbers between right and left ganglia, but in contrast to our findings, they reported that the right nodose ganglion had slightly more neurons than the left (6122 and 5211, respectively). We have no explanation for the difference between our results and those of Carobi et al., especially since we counted the same neuronal structures (nuclei). However, counts of the total number of fibers in the abdominal vagi of the rat by Precht and Powley (Precht and Powley, 1990) were higher in the ventral trunk (left) than in the dorsal trunk (right) (11327 and 10945 respectively). Assuming that the proportion of the afferent fiber population is reflected in the total number of fibers, we take this finding as support for our observation of slightly larger numbers of neurons in the left nodose ganglion. The significance of the differences in neuronal numbers will require additional investigation.

Expression of NR1 immunoreactivity or mRNA been reported in the cell bodies of the rat nodose ganglion (Shigemoto et al., 1992; Chang et al., 2003) and dorsal root ganglia (Marvizon et al., 2002). Also, an assessment of NR2 subunit expression has been reported for dorsal root ganglia (DRG) (Marvizon et al., 2002) but, to the best of our knowledge, no such assessment has previously been published for nodose ganglion. Our single and double immunofluorescent staining revealed that approximately 50% of NG neurons expressed NR2B and/or NR2C immunoreactivity, but only a minority of neurons in the nodose expressed NR2D immunoreactivity. NR2B, NR2C and NR2D NMDA receptor subunits co-expressed with NR1 have also been shown in DRGs (Marvizon et al., 2002). Marvizon et al (Marvizon et al., 2002) reported that 90% of DRG neurons express NR2B, while we found that only 56.7% of nodose neurons express NR2B. Furthermore, we found that more neurons express NR2C in the nodose than in DRG. Thus, while both the nodose and DRG express all of the NR2 subunits, there are marked differences between the proportions of neurons that express specific subunits, the NR2B subunit being much less prevalent in the visceral afferents of the nodose ganglion than among somatic afferents in the DRG.

Our observation that nearly all nodose ganglion neurons express NR1 immunoreactivity suggests that NMDA receptors participate in vagal afferent neurotransmission. However, since there are no synapses in the nodose ganglion itself, it seems probable that vagal afferent NMDA receptors participate in presynaptic modulation of signaling between primary vagal afferents and second order neurons in the nucleus of the solitary tract. In this regard, it is interesting that ultrastructural studies have revealed immunoreactivity for NR1 subunit in vagal afferent terminals, especially in the medial NTS (Aicher et al., 1999; Huang et al., 2000; Marvizon et al., 2002). Presynaptic modulation of glutamatergic vagal afferent transmission may be an important mechanism for modulating visceral afferent signaling to the NTS (Paquet and Smith, 2000; Tzingounis and Nicoll, 2004). While, the role of presynaptic NMDA receptors in modulation of primary vagal afferent transmission has not been investigated, electrophysiological data suggests that presynaptic NMDA receptors on the axon terminals of primary somatosensory neurons inhibit glutamate release in the spinal dorsal horn (Bardoni et al., 2004). Electrophysiological experiments also suggest the existence of presynaptic inhibition of primary afferent glutamate release in the NTS (Chen et al., 1999), but a role for NMDA receptors in that process has not been investigated.

The NR1 NMDA receptor subunit comprises the glycine binding site of the receptor, and is a component of all NMDA receptor channels (McBain and Mayer, 1994; Mori and Mishina, 1995; Qiu et al., 2005). However, the NR2 subunit family possesses the glutamate binding site (Mori and Mishina, 1995; Yamakura and Shimoji, 1999). Therefore, functional NMDA ion channels exist as heteromers of NR1 subunits with one or more NR2 subunit types (Mori and Mishina, 1995; Yamakura and Shimoji, 1999). Immunoreactivity and mRNA for the NR1 is widely distributed in the CNS (Akazawa et al., 1994; Boyce et al., 1999; Dingledine et al., 2001; Clayton and Browning, 2001; Cull-Candy and Leszkiewicz, 2004) and peripheral nervous system (Shigemoto et al., 1992; Burns et al., 1994; Marvizon et al., 2002; Chang et al., 2003). However, not all NR2 subunits are present in every NMDA channel heteromer. Hence, there appear to be a variety of NMDA channel phenotypes based, in part, on their NR2 subunit composition. Our results suggest that nodose ganglion neurons may express four to six distinct NMDA channel phenotypes based on the proportions of the three NR2 subunits for which we stained, and there is the potential existence of triheteromeric as well as diheteromeric channels. In this regard, it is necessary to point out that this estimate may well be an under estimate, as we did not attempt to identify NR2A or NR3 subunits, and had no way of separating splice variants of NR1 (Goebel and Poosch, 1999).

The functional significance of phenotypic diversity in vagal afferent NMDA channels is not known. However, work with recombinant NMDA channels indicates that the presence of

different NR2 subunits is associated with major functional differences amongst receptor phenotypes. For example, NR1/NR2B heteromers are subject to pronounced Mg^{++} blockade, whilst NR1/NR2C or 2D heteromers are relatively resistant to Mg^{++} blockade (Momiya et al., 1996), which one would predict would make them functional in less depolarized conditions. Heteromers containing the NR2B or NR2C subunits have inactivation times on the order of tens of milliseconds, whereas inactivation time for NR1/NR2D diheteromeric channels span several seconds (Monyer et al., 1994; Wyllie et al., 1998; Vicini et al., 1998). Hence, the length of time that NR1/NR2B or NR2C channels influence transmitter release from vagal afferent terminals might be much shorter than it is for NR1/NR2D.

DRG neurons have been reported to express two different NMDA receptors, one a diheteromer NR1/NR2B, found in both neurofilament-positive (A-type) and neurofilament-negative (C-type) neurons, plus bi- or triheteromeric (NR1-NR2D or NR1-NR2D-NR2C), channels present only on C-type neurons. In our study, most if not all NR2D subunit-containing neurons appear to express both NR2C and NR2B immunoreactivity. This suggests that, in the nodose ganglion, NR2D-containing NMDA receptors may exist in tetraheteromers. However, previous reports using this antiserum (Marvizon et al, 2002) suggest that this antibody also may recognize the NMDA receptor NR2C subunit. Thus, we can not rule out the possibility that our NMDA NR2D subunit staining was due to detection of the NR2C subunit. However, it is important to note that NR2C-IR neurons comprised a much larger population of nodose neurons ($49.4\% \pm 1.9\%$) than NR2D-IR neurons ($13.5\% \pm 2.0\%$). This difference in the prevalence of staining with these two antisera suggests that either the anti-NR2D antiserum was specific or that the antiserum recognized a subpopulation of neurons, which expressed especially high levels of NR2C-IR.

Direct injection of NMDA receptor antagonists into the dorsal vagal complex has been associated with a variety of systemic responses (Treece et al., 1998; Viard and Sapru, 2002; Antunes et al., 2004). However, none of these responses can be compellingly linked to antagonism of receptors on vagal afferent terminals or axons. However, Treece et al. (1998) have demonstrated that nanoinjection of the NMDA channel blocker, MK-801, directly into the medial NTS results increases food intake by delaying termination of an ongoing meal. Recent work by Hung et al. (in press) reveals a similar effect with NTS injection of the competitive NMDA receptor antagonist, APV. Interestingly, Gillespie et al. (2005) recently reported that the increased meal size produced by NTS MK-801 injection is abolished ipsilateral to nodose ganglion removal, but not ipsilateral to subnodose vagotomy. This finding supports participation by NMDA receptors on central vagal afferent terminals in control of food intake.

With the recognition of functionally distinct triheteromeric receptors, and possible tetraheteromeric receptors, the identification of the NR3 subunit family, and the added functional complexity conferred by NR1 splice variants, it is now apparent that the functional diversity of native NMDA receptors may be much greater than thought previously. Expression of NMDA receptors on presynaptic terminals of primary afferent neuron terminals of the vagal and spinal afferents suggests an important role for presynaptic NMDA receptors in the modulation of incoming sensory information. The physiological significance of heterogeneous, NMDA receptors on primary vagal afferents may ultimately be related to differences in the mechanisms by which distinct visceral afferent modalities are modulated. In addition, there were no obvious patterns to the distribution of dual-labeled neurons in the nodose ganglia. Determining whether the uniformly distributed or clustered neurons expressing NMDA receptor subunits in nodose ganglia exist as specific functional groups will require further study.

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Figure 1.

Photomicrographs of nodose ganglion neurons labeled with affinity-purified polyclonal primary antisera raised in goat against the NR1 (A), NR2B (B), NR2C (C), and NR2D (D) NMDA receptor subunits. Panels on the left (A, C) show stained neurons in longitudinal sections (20 μm) from the cranial (A,C) and caudal (B,D) portions of nodose ganglia. Scale bar = 50 μm .

Figure 2. Histogram showing the percentage of nodose neurons that express NMDA receptor subunit phenotypes.

Figure 3.

Longitudinal serial sections from the same nodose ganglion, separated by 40 μm , stained with affinity-purified polyclonal primary antisera raised in goat against the NR1 (A), and NR2D (B) NMDA receptor subunits. Note that NR1 and NR 2D-labeled neurons are fairly evenly distributed throughout the ganglia, whereas in some instances NR2C-IR perikarya were gathered into clusters of cells (C). Scale bar = 200 μm .

Figure 4. Photomicrographs of nodose ganglion sections double-labeled for NR2B (A) and NR2C (B) NMDA receptor subunits with primary antisera raised in rabbit (NR2B) or goat (NR2C). Double labeling immunofluorescence revealed co-localization of NR2B and NR2C subunit containing neurons (short arrows). Additionally, we found a subpopulation of NR2B subunit-containing neurons that did not express NR2C immunoreactivity (arrowheads) and NR2B immunonegative neurons expressing NR2C immunoreactivity (long arrows). Scale bar = 50 μ m.

Table 1

Primary antibodies

Target	Source	Catalog number	Host Species	Dilut ion	Immunogen Sequence	Specificity in western blot
NR1	Santa Cruz, CA	sc-1467	Goat polyclonal	1:100	NR1 subunit carboxyl terminus, amino acids 918–938	Single 115kDa band for pan-NR1 (Marvizon et al., 2002; SC)
NR2B	Santa Cruz, CA	sc-1469	Goat polyclonal	1:250	NR2B subunit carboxyl terminus, amino acids 1463–1482	Single 178 kDa band for NR2B (Marvizon et al., 2002; SC).
NR2B	Zymed Laboratories, CA	71-8600	Rabbit polyclonal	1:100	Fusion protein containing N-terminus amino acids 50–350 of NR2B subunit	Single 180 kDa band for NR2B (Zymed Lab.)
NR2C	Santa Cruz, CA	sc-1470	Goat polyclonal	1:500	NR2C subunit carboxyl terminus, amino acids 1218–1236	Single 160 kDa band for NR2C; blocked by NR2C but not NR2D immunogen, (Marvizon et al., 2002; SC).
NR2C	Novus Biologicals	NB 300-107	Rabbit polyclonal	1:200	Fusion protein containing N-terminus amino acids 25–130 of rat NR2C subunit	Single band for NR2C subunit at approximately 140 kDa (NB). Reportedly also stains 180 kDa band for NR2A/B in cerebellum.
NR2D	Santa Cruz, CA	sc-1471	Goat polyclonal	1:500	NR2D subunit carboxyl terminus, amino acids 1304–1322	Single 165 kDa band for NR2D, which is blocked by both NR2C and NR2D immunogens (Marvizon et al., 2002; SC).