The Journal of Experimental Biology 213, 2700-2709

doi:10.1242/jeb.041186

The importance of *N*-methyl-D-aspartate (NMDA) receptors in subtraction of electrosensory reafference in the dorsal nucleus of skates

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Accepted 21 April 2010

SUMMARY

The dorsal nucleus of the little skate is a cerebellum-like sensory structure that adaptively filters out predictable electrosensory inputs. The filter's plasticity is mediated by anti-Hebbian associative depression at the synapses between parallel fibers and ascending efferent neurons (AENs). Changes in synaptic strength are indicated by the formation of a cancellation signal which is initiated by co-activation of parallel fibers and AENs, and can be reversed by parallel fiber activity in the absence of AEN activation. In other cerebellum-like sensory structures, the formation of the cancellation signal requires activation of postsynaptic NMDA receptors on the principal neurons. We demonstrate here by immunohistochemistry that the somas and the initial portion of both apical and basal dendrites of the AENs are labeled with antibodies raised against the NR1 subunit of NMDA receptors from a South American electric fish. In *in vivo* physiological experiments, we show that the formation of the cancellation signal induced by coupling an electrosensory stimulus to ventilatory movements or direct parallel fiber stimulation is blocked when either of the NMDA receptor antagonists 2-amino-5-phosphonovaleric acid (APV) or MK801 is injected into the molecular layer above the recorded AEN. Blocking NMDA receptors prevented formation of a cancellation signal in 79% (15/19; APV) and 60% (3/5; MK801) of the AENs. This blockage was reversible in 40% (6/15) of the AENs after APV removal. Thus, in the dorsal nucleus, the activity-dependent, long-lasting but reversible change in synaptic strength of the parallel fiber–AEN synapses appears to be an NMDA receptor-dependent process.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/15/2700/DC1

Key words: cerebellum-like structure, NMDA receptor, synaptic plasticity, parallel fibers, learning and memory, electrosensory.

INTRODUCTION

The dorsal nucleus is the first stage for electrosensory processing in the central nervous system of little skates (*Raja erinacea*) and other elasmobranch fishes. As in the cerebellum-like structures of teleost electric fishes (Bell, 1981; Bastian, 1995), the dorsal nucleus removes reafference or other predictable features from incoming electrosensory inputs (Montgomery and Bodznick, 1994; Bodznick et al., 1999). During pairing of electrosensory stimulation with ventilation, fin movements or direct parallel fiber stimulation, a cancellation signal (negative image) is formed to remove reafferent information. This cancellation signal reflects the decrease and/or increase in the synaptic strength at the synapses between the parallel fibers and the principal neurons (ascending efferent neurons, AENs) (Bertetto, 2007). However, the molecular mechanisms underlying this plasticity in the dorsal nucleus are still unknown.

Glutamate is one of the principal excitatory neurotransmitters in the central nervous system of vertebrates. In adult mammalian cerebellum, glutamate neurotransmission at the parallel fiber synapses with Purkinje cells is mediated by AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) (Kano et al., 1988) and metabotropic glutamate receptors (mGluRs) (Knöpfel et al., 2000). However, the parallel fiber synapses in the cerebellum-like sensory structures, such as the electrosensory lateral line lobe (ELL) of mormyrid fish (Grant et al., 1998; Han et al., 2000; Berman et al., 2001) and dorsal cochlear nucleus (DCN) of some mammals (Manis and Molitor, 1996; Tzounopoulos et al., 2004; Tzounopoulos et al., 2007), are also mediated by NMDA (*N*-methyl-D-aspartate) receptors. The activation of NMDA receptors requires both presynaptic neurotransmitter release and postsynaptic depolarization to relieve the voltage-dependent Mg^{2+} blockage of the receptor channels (Nowak et al., 1984). The NMDA receptors thus can function as a 'coincidence detector' of presynaptic and postsynaptic activity. The unique biophysical properties and the important functions of NMDA receptors in various kinds of plasticity led us to test their role in the development of the cancellation signal responsible for suppressing electrosensory reafference in the dorsal nucleus of the little skates.

Here we demonstrate that the somas, apical dendrites and basal dendrites of the AENs in the peripheral zone of the dorsal nucleus are labeled by immunohistochemistry with antibodies specific to the NR1 subunit of NMDA receptors. In addition, we show that application of the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) blocked the formation of the cancellation signal in 79% (15/19) of the AENs. The cancellation signal was restored in 40% (6/15) of the AENs after APV removal. Another NMDA receptor antagonist MK801 appeared to block the formation of the cancellation signal in 60% (3/5) of the AENs. Thus in the dorsal nucleus, the activity-dependent, long-lasting but reversible change in synaptic strength of the parallel fiber–AEN synapses is consistent with an NMDA receptor-dependent process.

MATERIALS AND METHODS

Western blot

A primary antibody to the NR1 subunit of NMDA receptors was kindly provided by Dr Robert Dunn of McGill University (Bottai

et al., 1998). The antibody was prepared against the NR1 subunit of the South American electric fish *Apteronotus leptorhynchus*. The presence of the NR1 subunit in the dorsal nucleus was first tested by western blot, and then with immunoprecipitation and mass spectrometry. Little skates, *Raja erinacea* (Mitchill 1825), were collected in Long Island Sound near Millstone, CT, and maintained in artificial seawater tanks at 10–14°C. The skates were deeply anesthetized in 0.03% benzocaine and the dorsal nucleus was then cut out and homogenized in ice-cold Tris buffer. A portion of the skate liver was also homogenized for a negative control. Electrophoresis was done on polyacrylamide gels with 50 μg of protein per lane, followed by transfer to immunoblot membranes for reaction with anti-NR1 antibody (1:1000), secondary antibody [biotinylated goat anti-rabbit IgG (H+L), Vector Laboratories Inc., Burlingame, CA, USA; 1:220], streptavidin horseradish peroxidase (Vector Laboratories; 1:1000) and Sigma Fast® DAB peroxidase substrate tablet set (Sigma, St Louis, MO, USA), according to standard procedures.

Immunoprecipitation and mass spectrometry

We used this antibody to perform immunoprecipitation experiments from whole tissue extracts of the dorsal nucleus according to standard procedures, and analyzed the immunoprecipitates by reversed-phase electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), which was performed essentially as previously described (Lee et al., 2004) on an LCQ Deca XP Ion Trap instrument fitted with a packed tip column source for nanospray (ThermoFisher Scientific, Waltham, MA, USA) and coupled to a HP1100 binary pump system and degasser (Agilent Technologies, Santa Clara, CA, USA). For capillary column production, one end of a polyimide-coated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was manually pulled to a fine point of about 5 μm with a micro-flame torch. The columns were in-house packed to about 13 cm with C18 resin (5 μm, 200 Å Magic C18AQ, Michrom BioResources, Auburn, CA, USA), and samples were subsequently loaded, using a pneumatic pump (Brechbuehler, Spring, TX, USA) at a constant helium gas pressure of 1500 p.s.i. (~10,350 kPa). The mass spectrometer was configured so that three CID (collision-induced dissociation) scans followed each survey scan, and sequence databases were searched using SEQUEST software (Eng et al., 1994) embedded in the Bioworks (ThermoFisher Scientific) package accompanying the mass spectrometer.

DAB-ABC reaction

The skates were deeply anesthetized in 0.03% benzocaine and perfused transcardially with skate Ringer solution (pH 7.4), followed by perfusion with 4% paraformaldehyde in phosphate buffer (0.1 mol l^{-1} PB, pH 7.4 in all procedures). Brains were removed and cut transversely just in front of and just behind the dorsal nucleus. This block contained the dorsal nucleus, the dorsal granular ridge containing the granule cells for the dorsal nucleus, the caudal lobe of the cerebellum and most of the medulla. The blocks were postfixed with 4% paraformaldehyde overnight at 4°C and then embedded in gelatin (10% gelatin + 25% sucrose in 0.1 mol l^{-1} PB). The gelatin blocks were trimmed to the correct size, nicked for orientation, and then postfixed with 4% paraformaldehyde for 3 h and washed with 0.1 mol l^{-1} PB overnight at 4°C. Frozen 36 μm transverse sections were cut with a sled microtome and collected in chilled PB. All reactions were done with free-floating sections. The sections in their solutions were gently stirred on a shaker table during all procedures.

The brain sections were washed 4 times for 10 min each in 0.1 mol l^{-1} PB, pre-bleached with 3% H_2O_2 for 20 min, then washed 3 times for 10 min each in 0.1 mol l^{-1} PB, incubated in blocking buffer (10% normal goat serum, 0.25% Triton X-100, 1% bovine serum albumen in PB, 2 h, room temperature), and then transferred to primary antibody (anti-NR1 antibody, 1:2000) for 18 h at 4°C. The brain sections were washed 4 times for 10 min each in 0.1 mol l^{-1} PB and incubated in secondary antibody [biotinylated goat anti-rabbit IgG (H+L), Vector Laboratories; 1:200] for 4 h at room temperature, and then washed 5 times for 10 min each in 0.1 mol l^{-1} PB and incubated in ABC (Vectastain® Elite ABC peroxidase kits, Vector Laboratories) in PB overnight at 4°C. The brain sections were washed 7 times for 10 min each in 0.1 mol l^{-1} PB and reacted with Sigma Fast® DAB peroxidase substrate tablet set, and then washed 3 times for 10 min each in 0.1 mol l^{-1} PB, mounted and coverslipped.

Retrograde labeling of AENs and NR1 staining

A skate was anesthetized by immersion in 0.03% benzocaine (Sigma), and the brain was exposed by opening and folding back a small cranial flap. Dextran (Alexa Fluor® 488, 10,000 MW, Invitrogen, Carlsbad, CA, USA) was injected into the lateral mesencephalic nucleus as a paste on the end of a pin. The cranial flap was subsequently sealed with 3M Vetbond™ tissue adhesive (Henry Schein Inc., Melville, NY, USA) and zinc dental cement. Following postoperative periods of 14–21 days, the skate was re-anesthetized in 0.03% benzocaine and perfused transcardially with skate Ringer solution (pH 7.4), followed by 4% paraformaldehyde in 0.1 mol l^{-1} PB. Brains were removed and the brain slices were cut and collected as described above. All reactions were done with free-floating sections.

The brain sections were washed 4 times for 10 min each in 0.1 mol l^{-1} PB and incubated in blocking buffer (10% normal goat serum, 0.25% Triton X-100, 1% bovine serum albumen in PB) for 2 h at room temperature, and then transferred to primary antibody (rabbit anti-NR1 antibody, 1:1000) for 18 h at 4°C. The brain sections were washed 5 times for 10 min each in 0.1 mol l^{-1} PB and incubated in secondary antibody [Alexa Fluor® 568 goat anti-rabbit IgG (H+L), Invitrogen; 1:200] for 2 h at room temperature, washed 5 times for 10 min each in 0.1 mol l^{-1} PB, incubated in Hoechst (1:10,000) for 30 min at room temperature, and then washed 3 times for 10 min each in 0.1 mol l^{-1} PB, mounted and coverslipped.

In some experiments, monoclonal mouse anti-NR1 antibody (1:1000, Invitrogen no. 32-0500) was used for comparison with the rabbit anti-NR1 antibody against electric fish. The immunogen of this antibody (according to Invitrogen) is the fusion protein-containing sequence from the intracellular loop between transmembrane regions III and IV of NMDA receptor 1 (NMDAR1). The brain sections were washed 5 times for 10 min each after primary antibody staining and transferred to biotinylated horse anti-mouse secondary antibody (Vector Laboratories; 1:200) for 2 h at room temperature. After 5 washes of 10 min each, the brain sections were incubated in Alexa 568-conjugated streptavidin (Vector Laboratories; 1:200) for 1.5 h at room temperature, and then washed 5 times for 10 min each and incubated in Hoechst as described above.

Electrophysiology methods

Little skates (*R. erinacea*) were anesthetized with 0.04% benzocaine and surgical procedures were performed as previously described (Duman and Bodznick, 1996). The brains were exposed by removing the overlying cartilage, and decerebrated by diencephalic section. The skates were either injected with tubocurare (0.1 mg kg^{-1}, i.v.,

Sigma) to eliminate all movements, or partially paralyzed by destroying the spinal cord to leave only normal ventilatory movements intact. After surgery, the skates were transferred to a Plexiglas experimental tank filled with cold seawater (9°C), and rigidly positioned with a Plexiglas head holder to keep the cranial opening just above the water surface. A 0.1–0.4 l min^{-1} flow of seawater was directed into the mouth as an extra support to ventilation.

The electrophysiological experiments were carried out as previously described (Zhang and Bodznick, 2008). In brief, the AENs were identified by their antidromic responses to electrical stimulation of the contralateral lateral mesencephalic nucleus (LMN) and their extracellular unit activity was recorded by Pt–black-tipped indium microelectrodes (2–7 MΩ, 1–2 μm tip), and then filtered, amplified, acquired and analyzed using Spike2 software (CED, Cambridge, UK). A force transducer was placed against the skin over the branchial chamber of the fish to continuously monitor the ventilation in partially paralyzed fish.

The parallel fibers were electrically stimulated using a tungsten microelectrode in the dorsal granular ridge and a return electrode in the seawater. Based on the known topography of the dorsal granular ridge to dorsal nucleus projection (Conley and Bodznick, 1994), the location of the dorsal granular ridge stimulating electrode was chosen and optimized to elicit the largest evoked potential response from the dorsal nucleus recording site. The parallel fiber stimuli were delivered every 2 s, as 250 ms, 25 Hz trains of pulses; each pulse was 0.2 ms, 2–5 V. Excitatory electrosensory receptive fields of AENs were localized and stimulated by a DC step, 2–10 μV dipole electric field. In some cases, the dipole was coupled to ventilation for 10 min or longer. The interval between breathing cycles was about 2–3 s. The duration of a breathing cycle was around 1 s.

For drug injection, two-barrel pipettes were pulled to a fine tip, and then manually broken to a total tip diameter of 10–20 μm. One barrel was filled with 1 mmol l^{-1} glutamate (or 1 mmol l^{-1} NMDA), and the second barrel was filled with 1 mmol l^{-1} APV (or 1 mmol l^{-1} MK801, or 1 mmol l^{-1} saline as a negative control). The pipette was slowly advanced into the molecular layer of the dorsal nucleus directly above the AEN being recorded. The injection pipette was connected to a pressure injector (Picospritzer®III, Parker Hannifin Corp., Cleveland, OH, USA), the ejection duration was 100 ms, and the ejection pressure was usually 20 p.s.i. (~138 kPa). The proximity of the injection pipette to AENs was indicated by the latency of the increase in the neuron firing rate evoked by glutamate or NMDA injection. After appropriate adjustment, APV, MK801 or saline was delivered as a single pulse or a series of pulses (0.003–0.02 μl).

All procedures followed NIH guidelines for the care and use of experimental animals and were approved by the Animal Care and Use Committees of Wesleyan University and the Marine Biology Laboratory.

Data analysis

A cancellation signal was defined as a significant decrease in the AEN subtracted spike rates specific to the stimulation period after coupling an excitatory electrosensory dipole stimulus to the breathing movements for 5 or 10 min (Zhang and Bodznick, 2008). The subtracted spike rates were calculated as previously described (Zhang and Bodznick, 2008):

$$S_s = (S_t/T_t) - (S_o/T_o)\,, \quad (1)$$

where S_s is the subtracted spike rate (spikes s^{-1}), S_t is the number of spikes counted during the stimulation period, S_o is the number of spikes counted during the arbitrary control interval outside the stimulation period, T_t is the duration of the stimulation period in seconds and T_o is the duration of the arbitrary control interval outside the stimulation period in seconds. The AEN's response to one stimulation was defined as one trial. For each trail there was one S_s. A Wilcoxon–Mann–Whitney test was used to compare the change in AEN subtracted spike rate 40 trials (80 s time window) right before and 40 trials right after the coupling. Each AEN was analyzed independently.

RESULTS

The anti-NR1 antibody shows specificity in skates

The anti-NR1 antibody provided by Dr Dunn was produced using the carboxyterminal (intracellular tail segment) of *Apteronotus* NMDA receptor NR1 subunit protein. This segment is present in all forms of the NMDA receptor NR1 protein (Bottai et al., 1998). In our experiments, we tested the specificity of this antibody by both mass spectrometry and western blot to make sure that it was specific to the NMDA receptor in skates and could be used in the subsequent immunohistochemical experiments.

After immunoprecipitation, a band on the immunoblot membrane corresponding to a protein of about 100 kDa and reactive with the NR1-specific antibody was excised and digested with trypsin. It is worth mentioning that the NMDA receptor is a heterotetramer and is composed of two NR1 and two NR2 subunits; thus the immunoprecipitates may contain both NR1 and NR2 subunits. Interrogation of sequence databases with the tandem mass spectra acquired following processing of the band was performed to identify parent proteins. PeptideProphet software was used to estimate the accuracy of peptide assignments to tandem mass (MS/MS) spectra made by SEQUEST (Keller et al., 2002). Only robust matches, with PeptideProphet scores greater than 0.9, were considered. We identified peptides from the 100 kDa band robustly matching NR-related proteins from several vertebrate organisms, with the highest scores (PeptideProphet score=1) corresponding to NMDA receptors of Norway rat (*Rattus norvegicus*), NR2D subunits of zebrafish (*Danio rerio*) and NR2A subunits of cow (*Bos taurus*) and dog (*Canis familiaris*). The PeptideProphet score was 0.94 for the South American electric fish *A. leptorhynchus* (see Table 1).

Western blot analysis of whole dorsal nucleus extracts revealed a single band migrating at approximately 116 kDa, corresponding to the molecular mass of other known NR1 subunits. This band was absent in samples from the skate liver (Fig. 1A). This anti-NR1 antibody also recognized an approximately 116 kDa single protein in the cerebellum of the skate (not shown).

The results from both the mass spectrometry and western blot analysis demonstrate that the antibody is specific to the NMDA receptor NR1 subunit protein in little skates and suitable for detecting NMDA receptors in the immunohistochemical studies.

The distribution of NMDA receptor NR1 subunits in the dorsal nucleus

Different layers can be seen clearly on a transverse section of the dorsal nucleus after DAB reaction (Fig. 1B): the molecular layer, where the parallel fibers from granule cells of the dorsal granular ridge synapse on the apical dendrites of the AENs; the peripheral zone, where the somas of the principal neurons (AENs) are located; and the central zone, where the primary afferents synapse on the basal dendrites of the AENs.

In the molecular layer, the somas of stellate interneurons were heavily stained with anti-NR1 antibody (supplementary material Fig. S1A,B). However, we could not resolve any staining of the peripheral dendrites of the AENs or synaptic puncta in the molecular

Table 1. Identification of the antibody-labeled protein band by mass spectrometry and a sequence database search using SEQUEST software

PeptideProphet score	Protein probability	Percent coverage	Total number of peptides	Peptide sequence	Protein identification
1	1	0.6	1	RSEQEEVDVDR	NR2D (*Danio rerio*)
1	1	3.3	11	SSPHSEGSDR	NR2A (*Bos taurus*), NR2A (*Canis familiaris*)
	1	3.3	11	FGTVPNGSTER	
	1	3.3	11	HAVWPRYK	
	1	3.3	11	PSRSISLK	
1	1	1.7	1	EAMLRAAWAR	NR2C (*Rattus norvegicus*), NMDAR (*Rattus norvegicus*)
	1	1.7	1	FGTVPNGSTER	
0.98	0.98	2.6	4	RHKDA	NR1-4a (*Xenopus laevis*), NMDAR (*Xenopus laevis*)
	0.98	2.6	4	PTTSDGTCR	
	0.98	2.6	4	VLQFEPGTK	
0.96	0.96	2.2	2	FGTVPNGSTER	NR1 (*Gallus gallus*)
	0.96	2.2	2	DITERDIR	
	0.96	2.2	2	QESVRQGQGSQR	
	0.96	2.2	2	PSRSISLK	
0.96	0.96	1.3	1	PPAARPTGAPQPGK	NR3B (*Homo sapiens*)
0.96	0.96	3.6	4	SFAVTETLQMGIK	NR3A (*Homo sapiens*)
	0.96	3.6	4	THFHHPNK	
	0.96	3.6	4	VEKSRWR	
	0.96	3.6	4	ESSAEDYVR	
0.94	0.94	1.8	1	RHKDA	NR1 (*Apteronotus leptorhynchus*)
	0.94	1.8	1	EEHTPNGVLIKK	
0.93	0.93	1.2	1	ATVESIIEFEPK	NR1 (*Aedes aegypti*)

Only robust matches, with PeptideProphet scores greater than 0.9, were considered. The peptides from the 100 kDa band robustly match NMDA receptor-related proteins from several vertebrate organisms and the South American electric fish *Apteronotus leptorhynchus*.

layer. In the peripheral zone, the NR1 antibody labeled the somas and dendrites of neurons that, based on their morphologies, were presumed to be AENs and interneurons (Duman and Bodznick, 1997). The AEN-like neurons have spindle-shaped or multipolar somas about 30 μm in length and 10 μm in width (Fig. 1B,C). The interneurons have more spherically shaped somas about 10–20 μm in diameter. In the central zone, the anti-NR1 antibody labeled similar interneurons, which are 10–20 μm in diameter and have round soma. Some of these interneurons may also be commissural neurons (supplementary material Fig. S1A,C). We were not able to clearly see punctate staining indicative of synapses in peripheral or central zones. All labeling was absent when the primary antibody was omitted (Fig. 1D).

Retrogradely labeled AENs were also stained with anti-NR1 antibody

In order to confirm that the fusiform and multipolar cells of the peripheral zone that were labeled with the anti-NR1 antibody are

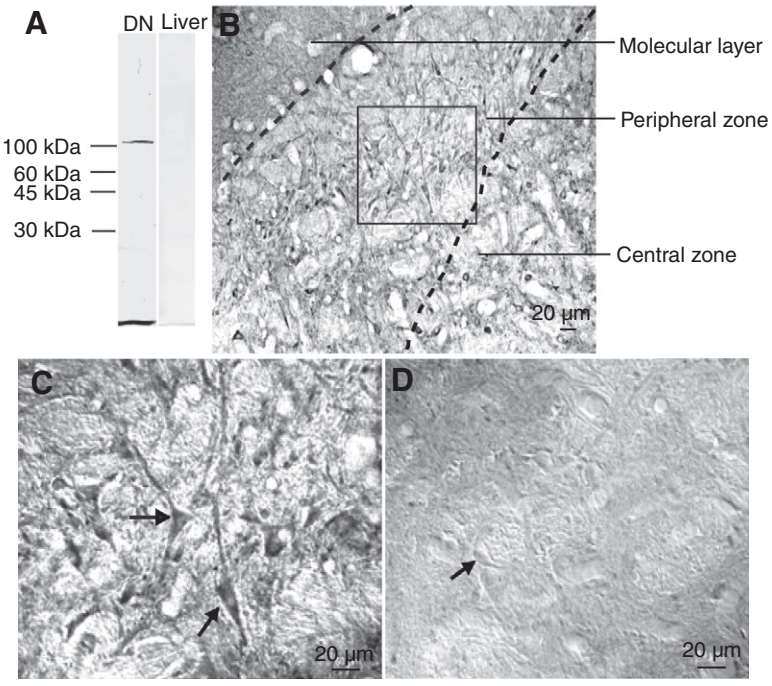

Fig. 1. The distribution of NMDA receptors in the dorsal nucleus (DN). (A) Western blot demonstrated that the anti-NR1 antibody recognized a single protein (116 kDa) in the dorsal nucleus. No signal was seen in the skate liver. (B) A coronal section of the dorsal nucleus labeled with the anti-NR1 antibody followed by DAB peroxidase staining. (C) High magnification of the area indicated in B. Ascending efferent neuron (AEN)-like neurons in the peripheral zone with spindle-shaped or multipolar somas, 30 μm in length, 10 μm in width, were labeled with anti-NR1 antibody (indicated by the arrows). (D) No staining is seen in a transverse section of the dorsal nucleus without anti-NR1 antibody as a negative control. The arrow points to an AEN-like neuron. Scale bars: 20 μm.

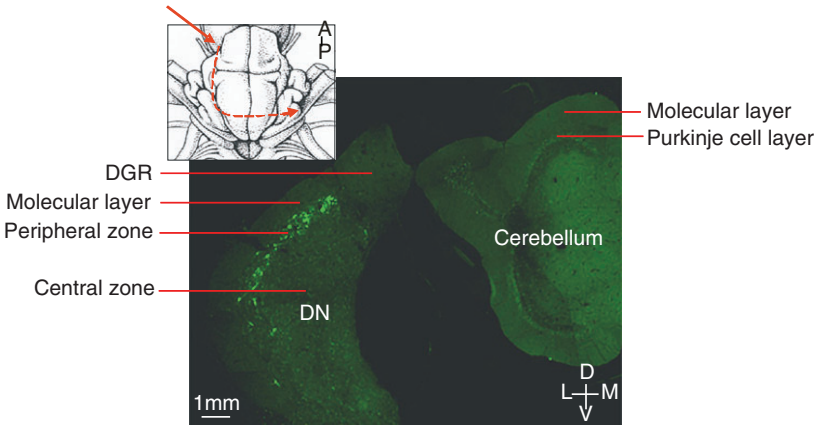

Fig. 2. A transverse section of the dorsal nucleus showing retrogradely labeled AENs in the peripheral zone following injection of dextran (Alexa Fluor® 488, 10,000 MW) into the contralateral lateral mesencephalic nucleus. The somas, the bases of apical and basal dendrites, and part of the proximal dendrites were filled with dextran. DGR, dorsal granular ridge; DN, dorsal nucleus; A, anterior; P, posterior; D: dorsal; V, ventral; L: lateral; M, medial. Scale bar: 1 mm.

indeed AENs, antibody staining was combined with retrograde labeling of the AENs in 10 animals. The AENs located in the peripheral zone were retrogradely labeled following the injection of dextran (Alexa Fluor®488, 10,000 MW) into the LMN, which is the principal midbrain target of the AENs. The somas and in some cases also part of the proximal dendrites of the AENs in the contralateral dorsal nucleus were filled with dextran (Fig. 2). It is worth mentioning that typically not all of the presumed AENs were retrogradely labeled with dextran, the number depending on the injection size and location, the dextran transfer rate and the fish's survival time. For those AENs that were retrogradely labeled with dextran, virtually all of them also appeared to be stained with the anti-NR1 antibody.

At the peripheral zone of the dorsal nucleus, the somas and the apical and basal dendrites of the retrogradely labeled AENs were stained with rabbit anti-NR1 antibody (Fig. 3A–D, Fig. 4). The NR1 antibody staining was in a punctate pattern. The staining which is present throughout the soma and bases of the dendrites was absent from the nuclei. The AENs have spindle-shaped or multipolar somas about 30 µm in length, 10 µm in width, which is consistent with the NR1 antibody staining using DAB (Fig. 1B,C; Fig. 4B). Labeling was absent when the primary antibody was omitted (Fig. 3E–H). These results demonstrate that the NMDA receptor NR1 subunits are located at the somas and the apical and basal dendrites of the AENs. Preliminary results using monoclonal mouse anti-NR1 antibody, made against a fusion protein containing sequence from the intracellular loop between transmembrane regions III and IV of mouse NMDAR1 (Invitrogen), gave a similar immunohistochemical staining pattern to the polyclonal rabbit antibody (not shown). There were no apparent differences between the monoclonal and polyclonal antibody staining patterns.

Blocking NMDA receptors prevents the formation of the cancellation signal

The AENs were identified by their antidromic response to electrical stimulation of the contralateral LMN. After coupling an external excitatory electrosensory stimulus to the animal's ventilatory movements or direct parallel fiber stimulation for 10 min or longer, a cancellation signal appeared in 62% (39/63) of the recorded AENs. The cancellation signal was apparent as a negative image of the response to the electrosensory stimulus when the stimulus was turned off. This is very similar to the proportion of AENs that have been found to exhibit plasticity when measured in this way in previous studies (Bodznick et al., 1999). Regardless of the stimulation protocol, 41% of these AENs (16/39) also displayed an incremental decrease in the response to the electrosensory stimulus (Fig. 5A,C), which is similar to results from previous studies (Zhang and Bodznick, 2008); the cancellation signal is the most robust indicator of the plasticity of the adaptive filter. However, the cancellation signals in some of the AENs, especially in the parallel fiber stimulation group (Fig. 6), were not as strong as in others. We do not know the explanation for this, but it is clear that our direct

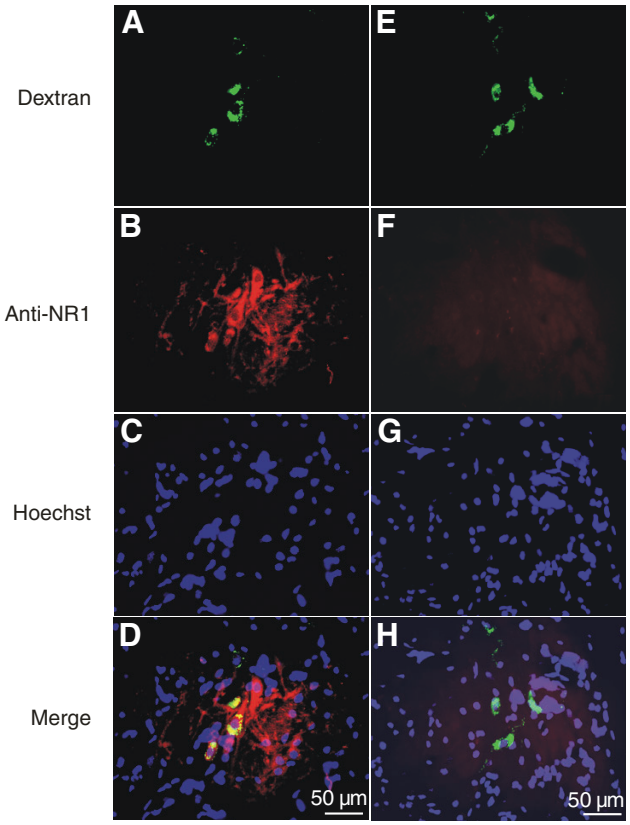

Fig. 3. Retrogradely labeled AENs were also stained with the anti-NR1 antibody in the dorsal nucleus. A–D are from the same section, E–H from a second section. (A,E) AENs in the peripheral zone of the dorsal nucleus retrogradely labeled with dextran (Alexa Fluor® 488, 10,000 MW). (B) Anti-NR1 antibody staining in the peripheral zone of the dorsal nucleus. The secondary antibody in this case is Alexa Fluor® 568 goat anti-rabbit IgG (H+L). (C,G) Hoechst staining. The nuclei of the cells in the dorsal nucleus were stained with Hoechst (1:10,000). (D) Merged image of A+B+C. The retrogradely labeled AENs were stained with rabbit anti-NR1 antibody. (F) A negative control. There was no staining in the absence of anti-NR1 antibody. (H) Merged image of E+F+G. Scale bars: 50 µm.

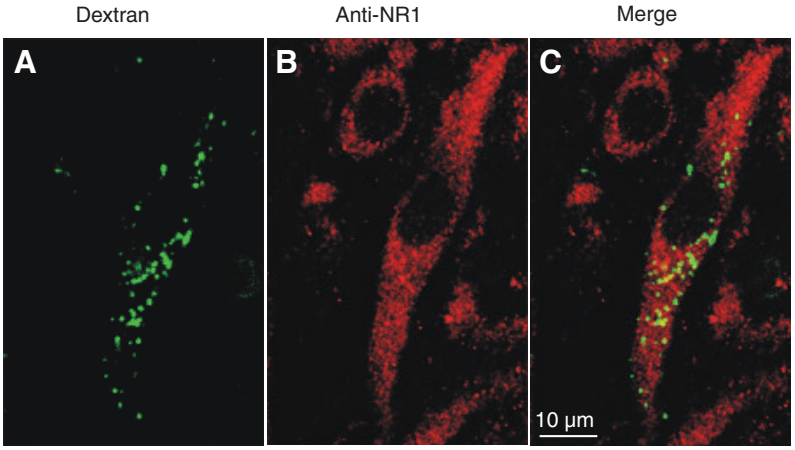

Fig. 4. Confocal images showing the antibody labeling of NR1 subunits of NMDA receptors on a retrogradely labeled AEN in the peripheral zone of the dorsal nucleus. (A) AEN retrogradely labeled with dextran in a punctate pattern. (B) Rabbit anti-NR1 antibody staining of the same section. The staining, which is present throughout the soma and bases of the dendrites, was absent from the nuclei. The cell on the left is possibly an AEN that was not retrogradely labeled by this particular injection into the lateral mesencephalic nucleus. (C) Merged image of A+B. Scale bar: 10 μm.

stimulation of the parallel fibers did not recreate the normal firing patterns among the parallel fibers that occur during natural behavior. In addition, the ongoing activity of parallel fibers associated with natural signals, such as ventilatory motor commands, could be antagonizing the plasticity being induced by direct parallel fiber stimulation of those same fibers.

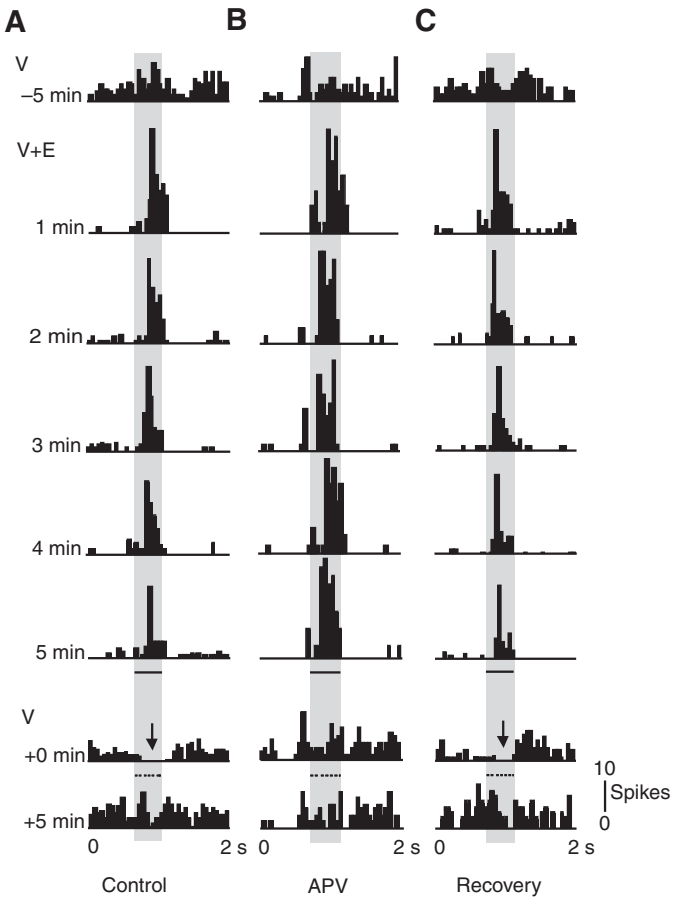

Fig. 5. The blockage of NMDA receptors by the NMDA receptor antagonist APV eliminated the formation of a cancellation signal. The formation of a cancellation signal (arrow) during the coupling of an external electrosensory stimulus to the skate's ventilatory movements (A) is blocked by APV injection (B). The formation of a cancellation signal reappeared after washout of APV (C). V, ventilation alone. V+E, 2 V local dipole stimulus coupled to ventilation. The solid line indicates the stimulus. The dotted line below the histogram indicates the period of the previous dipole stimulus. The arrow indicates the cancellation signal.

The 39 AENs that showed plasticity as evidenced by the presence of a cancellation signal after the coupling test were chosen for further testing. After the neuron activity returned to baseline level following the initial coupling test, 0.01–0.02 μl glutamate (1 mmol l^{-1}) or NMDA (1 mmol l^{-1}) was micro-pressure (20 p.s.i., 138 kPa) injected through a two-barrel pipette positioned in the molecular layer, where the parallel fibers synapse on the apical dendrites of the AENs. Only those AENs that responded to glutamate or NMDA injection in less than 60 s with a distinct increase in firing rate were chosen for further testing. The position of the injection pipette was adjusted to induce the strongest response; however, moving the injection pipette to a more favorable position for a given AEN most often resulted in loss of the cell. Only 24 out of 39 AENs were chosen or held sufficiently long for antagonist testing. Among the 24 AENs, 19 AENs were tested for APV injection and 5 AENs were tested for MK801 injection.

After the AEN activity had returned to baseline following glutamate or NMDA injection, 0.003–0.02 μl APV (1 mmol l^{-1}) was injected into the molecular layer to block the NMDA receptors located at the parallel fiber–AEN synapses. Within 5–10 min of APV injection, another coupling test was performed to determine whether the blockage of NMDA receptors would stop the formation of the cancellation signal. In 79% (15/19) of the AENs tested, APV injection eliminated formation of the cancellation signal (Fig. 5A,B). A coupling test was carried out approximately every 10 min to determine whether the cancellation signal was restored. In general it took 30 min to 2 h for the APV to be washed out. The cancellation signal was restored in 40% (6/15) of the AENs (Fig. 5C). In experiments in which MK801 was injected into the molecular layer, the blockage of NMDA receptors eliminated the formation of the cancellation signal in 60% (3/5) of the AENs (Fig. 6). MK801 is an irreversible antagonist; cancellation signals were not restored in any of the three AENs even after waiting for as long as 3 h. There was no significant change in the AEN baseline activity level (pre-pairing period) response magnitude (spike rate) after APV or MK801 application (Figs 5 and 6).

DISCUSSION

The NMDA receptor NR1 subunits are widely distributed in the dorsal nucleus of little skates

The dorsal nucleus in skates is an adaptive sensory processor and can generate cancellation signals (or negative images) of predictable features in sensory inputs. The cancellation signals can minimize the response to predictable features and make new and useful signals

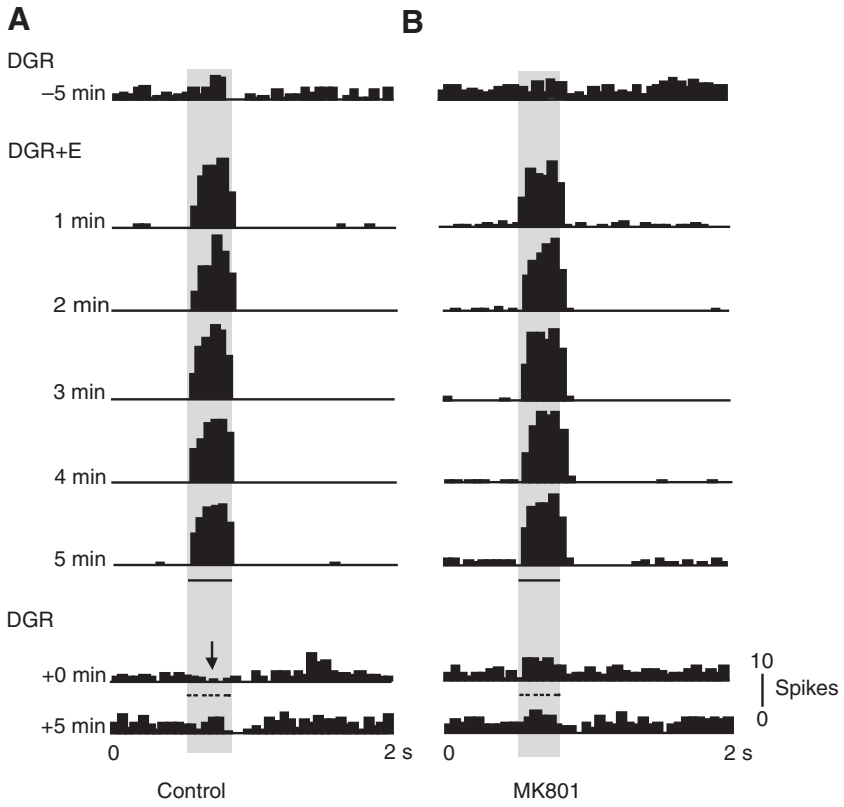

Fig. 6. The effect of blockage of NMDA receptors by MK801 on the formation of a cancellation signal. The formation of a cancellation signal (arrow) present after coupling an external stimulus to parallel fiber stimulation (A) is eliminated by MK801 injection (B). DGR, parallel fiber stimulation alone. DGR+E, 2 V local dipole stimulus coupled to parallel fiber stimulation. Other labels as in Fig. 5.

stand out (Montgomery and Bodznick, 1994; Bodznick et al., 1999). The studies done in elasmobranchs (Bodznick et al., 1996; Bodznick et al., 1999; Bertetto, 2007) and in the cerebellum-like structures known as the electrosensory lateral line lobes (ELLs) in mormyrid (Bell, 1982; Bell et al., 1997b) (for a review, see Bell et al., 1997a) and gymnotid (Bastian, 1995; Bastian, 1996a; Bastian, 1996b; Wang and Maler, 1997) electric fish, indicate that the generation of cancellation signals is largely due to plasticity at the synapses between parallel fibers and principal neurons. However, the cellular and molecular mechanisms underlying the plasticity in the dorsal nucleus are still unclear.

The excitatory neurotransmitter at the parallel fiber–AEN synapses of the skate dorsal nucleus is presumed to be glutamate as it is in other cerebellar-like structures and the cerebellum. It is well known that, as one of the glutamate receptors, NMDA receptors have specific biological properties that suit them to a role as coincidence detectors. The activation of the receptors requires both binding of glutamate to the receptor and significant postsynaptic depolarization to relieve the Mg^{2+} block. The co-activation of parallel fibers and depolarization of AENs is required for generating a cancellation signal (Bertetto, 2007). NMDA receptors, if present, would be particularly suitable for detecting such coincident activity and mediating associative depression at parallel fiber synapses; calcium influx should occur predominantly when parallel fibers are active while AENs are depolarized.

By using immunohistochemistry directed at the NR1 subunit of NMDA receptors, we examined the existence and distribution of NMDA receptors in the dorsal nucleus of the little skates. As demonstrated in Fig. 1, the NMDA receptor NR1 subunits are located at different layers of the dorsal nucleus. Not only the AEN-like neurons but also interneurons in both the peripheral and central zones were labeled with anti-NR1 antibody. Using DAB staining, we could only identify the presumed AENs by their location, shape and size. For more accurate identification, we further combined retrograde labeling of AENs with NR1 staining. The results demonstrate staining of the somas and the apical and basal dendrites of the AENs with anti-NR1 antibody. Similar staining patterns are seen in the ELLs of mormyrid and gymnotid fish (Bell et al., 2005; Berman et al., 2001). In the mormyrid and gymnotid experiments, the antibody staining was sufficiently intense and complete to demonstrate that the spines at the apical dendrites of the principal neurons are stained with anti-NR1 antibody (Bell et al., 2005; Berman et al., 2001). Theoretically, NMDA receptors located at the spines on the apical dendrites of the AENs in skates are critical for the plasticity in the adaptive filter; however, the staining was insufficient to demonstrate their presence there in the current study.

Variation in the effects of NMDA receptor antagonists

As described in the Results, the injection of APV eliminated the formation of the cancellation signal in 79% (15/19) of the AENs. In the other four AENs, the injection of APV had no detectable effect on the formation of the cancellation signal. Similar results were seen with MK801. Why did individual AENs respond differently to the NMDA receptor antagonist?

One explanation is that other receptors, such as mGluRs, may also be involved in the formation of cancellation signals in the dorsal nucleus. In other systems mGluR activation has been found to complement NMDA receptor-mediated plasticity. For instance, in the barrel cortex layer 4-2/3 synapses of mice, mGluR activation is required for further synaptic strengthening after the initial induction of long-term potentiation (LTP) by the activation of NMDA receptors (Clem et al., 2008). However, at excitatory synapses in the cerebellum (D'Angelo et al., 1999), hippocampus (Bashir et al., 1993) and amygdala (Lee et al., 2002), and some types of interneurons (Perez et al., 2001), mGluRs also support the

induction of LTP. In the dorsal nucleus, if both NMDA receptors and mGluRs are involved in the formation of the cancellation signal, the effect of blockage of the NMDA receptors might depend on how much the mGluRs contribute to the formation of the cancellation signal. However, this is only speculation as we currently know nothing of the presence or distribution of mGluRs in the skate dorsal nucleus and we have no *a priori* reason to expect the relative contributions of NMDA and mGlu receptors to vary among AENs.

Variations in the effectiveness of the blockage of NMDA receptors among AENs seems to us a more likely explanation of the variability in our results. This experiment was done *in vivo* where the antagonists were injected locally. It is impossible to measure the final concentration of the antagonists at the relevant receptors, or how effective and long lasting the blockage might be. This contrasts with similar experiments *in vitro* where the antagonist concentration throughout the tissue can be controlled through bath application. The position of the drug injection pipettes was adjusted based on each neuron's response to glutamate or NMDA injection. However, because APV (or MK801) and glutamate (or NMDA) have different pharmacological dynamics, it is possible that the NMDA receptor antagonists did not completely block the NMDA receptors located at the parallel fiber–AEN synapses when the recordings were carried out. Incomplete blockage of the NMDA receptors may be responsible for the different responses to the NMDA receptor antagonists injected in different AENs. We chose to do the experiments *in vivo* in order to be able to test the effects of blocking NMDA receptors on the synaptic plasticity in its functional context. The cost is less control and perhaps the resulting variability in outcome.

Other differences among the little skates, such as the fish's age and health condition, and anatomical differences in the brain, could also cause the difference in the response to APV injection. For example, more vasculature or variations in blood flow might cause differences in the responses to antagonist injection.

The NMDA receptors are important in the formation of cancellation signals

Cerebellum-like sensory structures in elasmobranchs, and in mormyrid and gymnotiform teleost fishes act as adaptive filters that can remove predictable features in the sensory inputs (Bell, 2001). The removal of the predictable features is indicated by the formation of cancellation signals (or negative images), which are the result of anti-Hebbian plasticity at parallel fiber synapses with the principal neurons (Bell, 2001).

In the mormyrid and gymnotid ELL, earlier studies demonstrate that the NMDA receptors play a role in synaptic plasticity at parallel fiber–principal neuron synapses (Han et al., 2000; Lewis and Maler, 2002). Although the dorsal nucleus and the ELLs are both so-called cerebellum-like sensory structures, the electrosensory systems in elasmobranchs and teleosts and thus also the dorsal nucleus and ELLs appear to have evolved independently (Bell, 2002).

To test whether NMDA receptors also play a role in the plasticity of the dorsal nucleus, we carried out a series of experiments. As the results indicate, the blockage of NMDA receptors eliminated the formation of cancellation signals. Thus, like other cerebellum-like sensory structures, activation of NMDA receptors appears to be important for the associative depression of parallel fiber–AEN synapses observed in the AENs.

In this *in vivo* experiment, when an external stimulus was coupled to the fish's ventilation (a natural behavior), the blockage of NMDA receptors in most cases stopped the formation of the cancellation signal. This indicates that synaptic weakening occurring during normal behavioral experience may require NMDA receptors.

The associative depression at parallel fiber–principal neuron synapses in cerebellum-like sensory structures is very similar to that in the cerebellum. It is undeniable that these parallel fiber systems are remarkably similar and certainly have some evolutionary and/or developmental relatedness (Sawtell and Bell, 2008) (for a review, see Bell et al., 2008). However, the cellular mechanisms underlying the synaptic plasticity appear to have distinct and fundamental differences. In the mormyrid and gymnotid ELL (Han et al., 2000; Lewis and Maler, 2002), the DCN in mammals (Tzounopoulos et al., 2004; Tzounopoulos et al., 2007), and – based on the results in this research – the dorsal nucleus in the elasmobranchs, the NMDA receptors play a role in synaptic plasticity at parallel fiber synapses onto principal neurons.

NMDA receptor antagonists can inhibit cell firing in the visual system (Miller et al., 1989; Sillito et al., 1990; Blitz and Regehr, 2003) and in the hippocampus (Abraham and Mason, 1988; Burgard et al., 1989; Dahl et al., 1990). Interestingly, in this study, blocking NMDA receptors did not change the AEN baseline firing activity. One possibility is that NMDA receptors coexist postsynaptically with other types of glutamate receptors (for a review, see Mayer and Westbrook, 1987), and both NMDA and non-NMDA receptors may mediate excitatory synaptic transmission at parallel fiber–AEN synapses. Non-NMDA receptors, such as AMPA receptors, mediate the vast majority of fast excitatory synaptic transmission (for a review, see Dingledine et al., 1999). If the non-NMDA receptor-mediated synaptic transmission was sufficiently large, blocking the NMDA receptors might not necessarily alter action potential firing (Zhao et al., 2005). Therefore, blocking of NMDA receptors can abolish the associative depression but have less impact on the background firing rate.

Although we think it is unlikely, it is also possible that the synaptic plasticity at parallel fiber–AEN synapses, just like the parallel fiber–Purkinje cell synapses in the cerebellum (Ito, 2001), is NMDA receptor independent. In the cerebellum, nitric oxide synthase (NOS) is activated by parallel fiber stimulation and nitric oxide (NO) is released to the postsynaptic Purkinje cells (Shibuki and Kimura, 1997). NO activates a cascade involving the activation of guanylyl cyclase and cyclic GMP, protein kinase G, and the phosphorylation of G-substrate. This cascade is NMDA receptor mediated and is required for long-term depression (LTD) induction in Purkinje cells (Ito, 2006). However, this NMDA receptor/NO cascade involved in cerebellar LTD is not located in parallel fiber terminals but is localized in stellate cell terminals (Shin and Linden, 2005). Parallel fibers form excitatory synaptic connections with stellate cells and stellate cells, in turn, form $GABA_A$ receptor-mediated inhibitory synapses on Purkinje cell dendrites and modulate Purkinje cell activity (van Vreeswijk and Sompolinsky, 1996). In the current experiment, other than in AENs, apparent NR1 labeling was also detected in stellate interneurons. It is possible that APV blocks LTD in the dorsal nucleus through an action on the interneurons as in the cerebellum. In future work, it will be important to identify the precise location of NMDA receptors in the molecular layer synapses in the dorsal nucleus, in order to see whether the dorsal nucleus is more like the other cerebellar-like structures or like the cerebellum. Meanwhile, the glutamate (Glu) receptor δ2 is involved in the plasticity at Purkinje cell synapses (Hirano et al., 1995); it will be interesting to find out whether the GluRδ2 is present and also contributes to the synaptic plasticity in cerebellum-like sensory structures.

CONCLUSIONS

The distribution of NMDA receptors in the dorsal nucleus was demonstrated using immunohistochemical methods. We demonstrated that the soma, apical dendrites and basal dendrites of the AENs in the peripheral zone of the dorsal nucleus can be labeled with antibodies specific to the NR1 subunit of NMDA receptors.

The functional significance of NMDA receptors was tested electrophysiologically. Each AEN served as its own control. A coupling test was first done in the absence of the NMDA receptor antagonist APV or MK801, and then couplings were done in the presence of APV or MK801.

By coupling an electrosensory stimulus to ventilation or parallel fiber stimulation, APV was found to block the formation of a cancellation signal in 15 out of 19 AENs. The cancellation signal was restored in 6 out of 15 of the AENs after APV removal. The injection of MK801 blocked the formation of a cancellation signal in 3 out of 5 AENs. Thus in the dorsal nucleus, the activity-dependent, long-lasting but reversible change in synaptic strength of the parallel fiber–AEN synapses appears to be an NMDA receptor-dependent process.

LIST OF ABBREVIATIONS

AEN	ascending efferent neuron
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	2-amino-5-phosphonovaleric acid
DCN	dorsal cochlear nucleus
ELL	electrosensory lateral line lobe
LMN	lateral mesencephalic nucleus
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
PB	phosphate buffer

ACKNOWLEDGEMENTS

We would like to thank Dr Stanley Lin for giving us insightful suggestions on this research; Dr Robert Dunn for kindly providing the anti-NR1 antibody; Dr Mark Flory for helping us with mass spectrometry; and Dr Janice Naegele for providing lab resources. We would like to thank Mr Billy Klem, Mr Danny Sullivan and Mr Eddie Enos at the Marine Biological Laboratory and the crew of the Environmental Lab at the Dominion Nuclear Plant in CT for collecting skates. This work was supported by an NSF grant to D.B.

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