Increased Expression of *N*-Methyl-*D*-Aspartate Receptor Subunit 2D in the Skin of Patients with Fibromyalgia

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ABSTRACT. ***Objective.*** We studied the expression of *N*-methyl-*D*-aspartate receptors (NMDAR) in skin of patients with fibromyalgia (FM) to investigate their role.

Methods. The presence of NMDAR subtype 2B (NR2B) and subtype 2D (NR2D) was examined in skin tissues by immunohistochemistry and immunoblot. Skin tissues from 11 female patients with FM were examined and compared to those of 8 age- and sex-matched healthy controls.

Results. NR2D expression was increased in the skin of patients with FM versus controls. There was no difference in the expression of NR2B between FM patients and controls.

Conclusion. The increased expression of NMDAR found in FM skin could be indicative of a more generalized increase in other peripheral nerves. This suggests that NR2D-selective antagonists may have implications in the treatment of allodynia in patients with FM. (J Rheumatol 2006;33:785–8)

Key Indexing Terms:
FIBROMYALGIA N-METHYL-D-ASPARTATE RECEPTORS SKIN

Fibromyalgia (FM) is a chronic pain disorder commonly seen in women, characterized by widespread muscular pain, tenderness, fatigue, unrefreshing sleep, and many other associated symptoms[1]. The pathogenesis of FM is not known, but much research has been carried out in FM to better understand the neurobiology of chronic pain in these patients. Some research suggests that dysregulated pain modulation appears to play an important role in FM[2].

Dorsal horn neurons of pain-related pathways undergo central sensitization during tonic impulse input from C nociceptive afferent neurons, and this phenomenon is, in turn, closely related to a slow temporal summation of activity termed "windup"[3,4]. This temporal summation or windup is dependent on *N*-methyl-*D*-aspartate receptors (NMDAR) and substance P synaptic mechanisms within the dorsal horn of the spinal cord[5]. Abnormal sensitization and temporal summation of second pain (windup) have been described in patients with FM[6].

Glutamate is a major excitatory amino acid neurotransmitter in the central nervous system, and its receptors can be classified as either ionotropic or metabotropic. Ionotropic glutamate receptor subtypes include the following 3 subtypes: NMDA, α-amino-3-hydroxy-5-methylisoxazolone-4-propionic acid, and kainite. Recent studies indicate that glutamate and its ionotropic receptors play a role in peripheral nociceptive transmission. These 3 receptor subtypes have been anatomically localized in dorsal root ganglion (DRG) cell bodies[7], and on peripheral primary afferent axons in hairy and glabrous skin in rats[8]. Recent psychophysical behavioral studies suggest the presence of glutamate receptors on peripheral primary afferents in humans[9]. Until now only one study has shown the presence of ionotropic glutamate receptors in cutaneous nerves in human hairy skin[10]. Peripheral nociceptive fibers express NMDAR subtype 2B (NR2B) and NMDAR subtype 2D (NR2D), whereas NMDAR subtype 2A (NR2A) appears to be absent from the peripheral terminals of primary afferents[11,12].

We studied the expression of NMDAR in the skin of patients with FM. We show evidence that the expression of NR2D is specifically increased in the skin of patients with FM.

MATERIALS AND METHODS

Patients and tissue samples. Skin tissues from 11 female patients with FM were examined and compared to skin biopsies of 8 age- and sex-matched healthy controls. Biopsies were obtained from the left deltoid region. The patients were diagnosed with FM according to the American College of Rheumatology criteria1[13]. To relieve FM symptoms they were taking drugs such as acetaminophen, tramadol, amitriptyline, and fluoxetine, but no drugs (e.g., NMDAR blockers) that could theoretically lead to increased expression of NMDA. The study had the approval of the ethics committee, and subjects were informed orally and in writing about the biopsy procedure and gave written informed consent. Skin tissues were obtained by punch biopsy of the anesthetized left deltoid region. Immediately after the procedure, a section of each tissue was snap-frozen in liquid nitrogen and stored at –70°C for immunoblot analysis.

Immunohistochemical analysis. Serial sections of 4 μm thickness were made and spread on poly-L-lysine coated slides. Paraffin sections were immersed in 3 changes of xylene and hydrated using a graded series of alcohol. Antigen retrieval was performed routinely by immersing the sections in 0.01 M citrate buffer (pH 6.0) in a pressure cooker by autoclaving for 15 min.

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Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min and then incubated with a primary antibody overnight in a humidified chamber at 4°C. Primary antibodies were goat polyclonal anti-NR2D (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-NR2B (1:2000) antibodies[14]. Staining was achieved with a Dako LSAB+ kit and developed with diaminobenzidine tetrahydrochloride (Dako, Carpinteria, CA, USA). Sections were counterstained for 5 min with Meyer's hematoxylin and then mounted. Rat DRG with intense staining for NR2D was used as a positive control. As a negative control, rabbit and goat IgG isotypes (Dako) were used instead of primary antibodies. Immunoreactivity was evaluated by assessing staining intensity and grading as absence (no staining), mild, moderate, strong, or strongest. Five immunohistochemical staining grades were assigned to each 0, 0.25, 0.5, 0.75, and 1 for statistical analysis.

Immunoblot. Tissues were homogenized in the gel-loading buffer [3.0% SDS, 5.0% glycerol, 2.0% b-mercaptoethanol, 62 mM Tris-Cl (pH 6.7)] and boiled for 5 min. About 40 μg of each sample were electrophoresed in 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred to a nitrocellulose membrane. After incubation of the blots overnight at 4°C in TTBS [0.2% Tween-20, 10 mM Tris–HCl (pH 7.5) and 0.2 M NaCl], goat polyclonal anti-NR2D (1:1000; Santa Cruz) was added and further incubated for 2 h at room temperature. Blots were rinsed in TTBS (4 × 20 min), and the antigen–antibody complex was visualized with alkaline phosphatase-conjugated anti-goat secondary antibody (1:2000; Santa Cruz). For quantification, blots were scanned to acquire a digital image. The signal intensities were measured using image analysis software (US National Institutes of Health Scion Image Beta 4.0.2) and were expressed throughout as the mean ± standard deviation.

Statistical analysis. Data were analyzed using Mann-Whitney U test and Fisher's exact test. Associations were estimated using Pearson's correlation. P values less than 0.05 were considered statistically significant.

RESULTS

Peripheral nerve fibers did not show any inflammatiory or degenerative changes in either of the groups. We performed immunohistochemical staining in each of the 5 tissue samples containing peripheral nerve fibers in both groups. NR2D staining intensity was higher in patients than in the control group ($p = 0.016$). Its expression was clearly seen in the peripheral nerve fibers of patients with FM (Figure 1). Density of NR2D was also increased in the FM patient pool compared to the control pool on immunoblot (Figure 2). Expression of NR2B was seen in peripheral nerve fiber but was similar between patients and controls (Figure 1).

Within the FM group, there was a correlation between levels of the NMDAR and disease duration ($p = 0.046$), but not between levels of the NMDAR and degree of tenderness (tender point number).

DISCUSSION

Our study demonstrates the increased expression of NR2D in FM skin. Glutamate and its ionotropic receptors are implicated in peripheral nociceptive transmission. Recently, it has been shown that peripheral nociceptive fibers express NR2B and NR2D, whereas NR2A appears to be absent from the peripheral terminals of primary afferents[11,12]. Therefore, we investigated the expression of NR2B and NR2D.

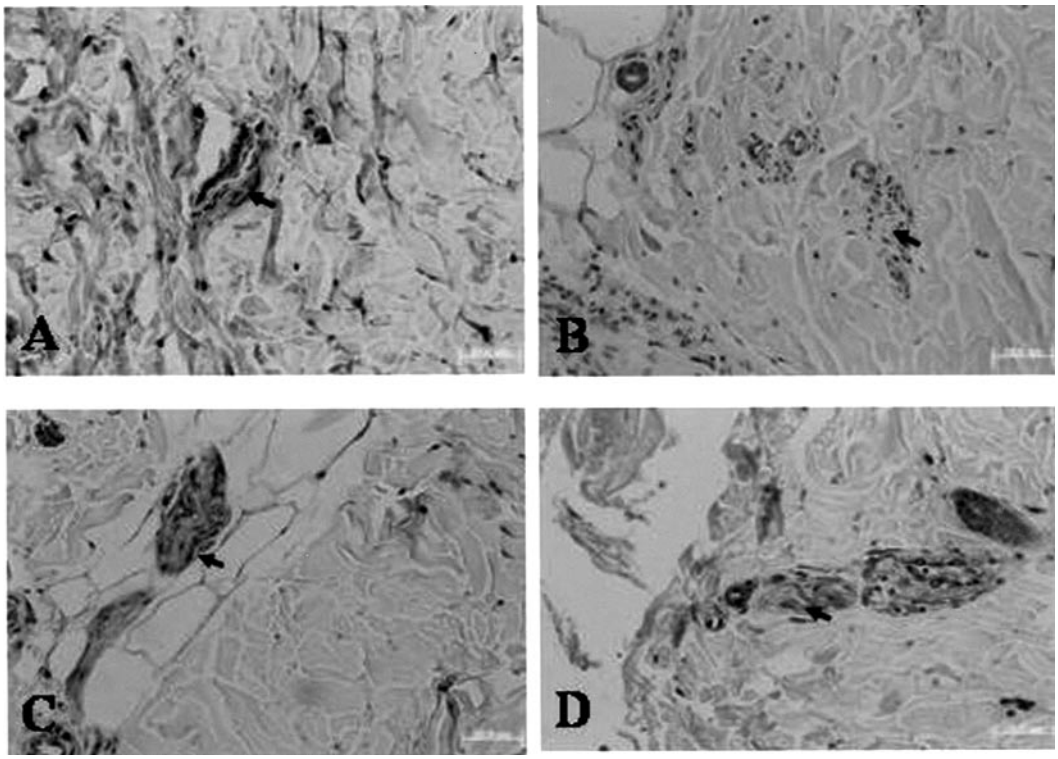

Figure 1. Immunohistochemical staining of NR2D (A, B) and NR2B (C, D) in patients (A, C) and controls (B, D). Patients show increased NR2D expression in peripheral nerve fiber (arrow), whereas the controls show weak staining. Expression of NR2B was seen in peripheral nerve fiber (arrow) but was similar between patients and controls. Magnification ×400.

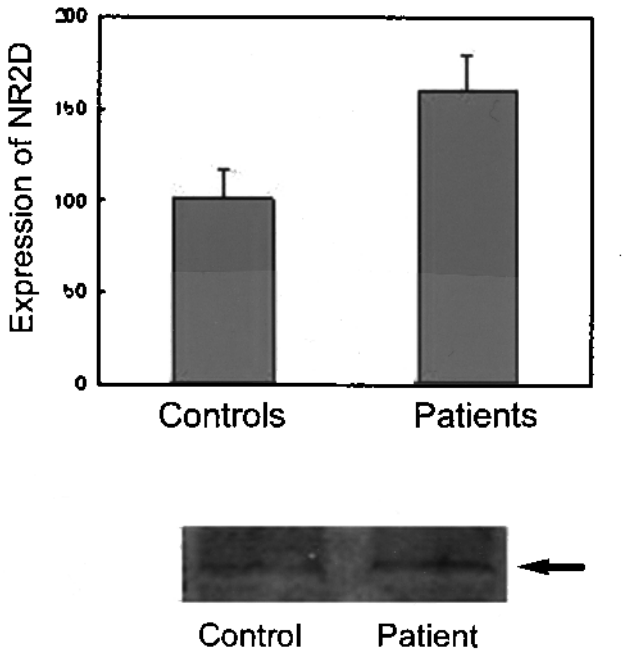

Figure 2. Representative immunoblot. Increased NR2D density of the FM patient pool versus the healthy control pool (159.01 vs 100.00). Data are given as arbitrary units.

In the last decade, the molecular biology of the NMDAR family has been defined, and we now know that these receptors are composed of an NMDAR subunit 1 (NR1) in combination with one or more NMDAR subunit 2 (NR2)[15]. The NR2, of which there are 4 (A–D), determine the pharmacology and other properties of NMDAR. There are differences in subunit expression. Whereas NR1 and NR2A are ubiquitous, NR2B is restricted to the forebrain, 2C to the cerebellum, and NR2D is much rarer than other subtypes. There is substantial evidence that the NMDAR play an important role in nociception. NMDAR are present in primary afferents, where they may play crucial roles in both peripheral and central sensitization[16]. Immunohistological stainings of rat DRG with specific antibodies revealed that DRG neurons have 2 different NMDAR, one containing the NR1, NR2D, and possibly the NR2C subunits, found only in C-fibers, and the diheteromer NR1/NR2B, present in the Golgi apparatus of both A- and C-fibers[12]. C-fibers are unmyelinated primary afferent fibers of cutaneous origin and have long been linked to nociception and pain, although that is not the sole afferent function of this population. Our data indicated that the expression of NR2D is significantly increased in patients. NR1/NR2D receptors have a much longer offset decay, lower conductance, higher affinity for glutamate, and weaker Mg^{2+} block than other NMDAR, but have comparable Ca^{2+} permeability. There is no evidence for NR1/NR2D receptors at any central synapse, but they have been identified in the extrasynaptic membrane of several cell types[17]. This extrasynaptic localization of NR1/NR2D receptors would be consistent with their presence on the peripheral terminals of primary afferents, where they may be involved in nociceptive transmission[18]. Because NR2D-containing NMDAR have a considerably long deactivation time constant (several seconds)[17], a small increase in this NMDAR subtype would result in a large increase in Ca^{2+} influx. This may explain why NR2D is involved in peripheral sensitization.

Our study is the first report to describe increased expression of NR2D in the skin of patients with FM. The increased expression of NR2D may contribute to peripheral sensitization in some patients with FM.

This increased expression of NMDAR in FM skin could be indicative of a more generalized increase in other peripheral nerves. It is clear that NMDAR are critically involved in the induction and maintenance of neuronal hyperexcitability. Until recently, only central NMDAR were a primary focus of investigations in FM. With the recognition of peripheral somatic and visceral NMDAR, it is now apparent that the role of NMDAR in pain is much greater than previously thought. Given the small side effect profile and good efficacy of NR2D-selective compounds, it is quite likely that NR2D-selective blockade will emerge as a viable strategy for pharmacological treatment of pain in FM.

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