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NMDA-R1 antisense oligodeoxynucleotides modify formalin-induced nociception and spinal c-Fos expression in rat spinal cord

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Abstract

Noxious peripheral stimuli (thermal, mechanical, or chemical) produce long-term adaptations in the sensitivity of central nociceptive neurons to subsequent noxious stimuli. The mechanisms responsible for this central sensitization are multifactorial, but the activation of spinal *N*-methyl-D-aspartate (NMDA) receptors plays a pivotal role. Using antisense oligodeoxynucleotides, we tested the role of the NR1 subunit of the NMDA receptor in the nociception and expression of the immediate early gene c-*fos* following formalin-induced pain. Rats received NMDA-R1 antisense, sense, or missense oligodeoxynucleotides intrathecally three times over a 48-h interval. The day after the last injection of the oligodeoxynucleotide, the formalin test was performed. Pain-related behavior was quantified by counting the incidence of flinching of the injected paw for 60 min, and the animals were perfused and the spinal cord removed for c-Fos immunohistochemistry 60 min later. Immunopositive cells were counted in the laminae I/II_0 and V of the lumbar enlargement. Treatment with NR1 antisense oligodeoxynucleotide resulted in a marked decrease in flinching. Similarly, the antisense oligodeoxynucleotide virtually abolished formalin-induced expression of c-Fos-like immunoreactivity (Fos-IR) in the spinal cord dorsal horn ipsilateral to injection. In contrast, the corresponding sense or missense oligodeoxynucleotides had no effect on either formalin-evoked behavior or c-Fos immunoreactivity. We conclude that an NR1 antisense oligodeoxynucleotide inhibits both nociceptive behavior and c-*fos* expression following formalin injection in rats, demonstrating that NR1 plays an important role in the development of noxious stimulation induced c-*fos* expression in this model.

Keywords: Nociception; Hyperalgesia; *N*-methyl-D-aspartate; Antisense oligodeoxynucleotide; Formalin test; c-Fos; Spinal cord; Intrathecal

1. Introduction

Spinal glutamate receptors play an important role in the facilitation and maintenance of nociception. AMPA and kainate receptors mediate acute nociceptive responses and monosynaptic reflexes, whereas *N*-methyl-D-aspartate (NMDA) receptors mediate polysynaptic reflexes and chronic nociceptive responses. Direct application of the NMDA receptor agonist glutamate to spinal nociresponsive neurons mimics the hyperalgesic responses seen with repetitive C-fiber stimulation. In addition, NMDA receptor antagonists profoundly inhibit the tonic phase (phase 2) of formalin-induced nociception (Munglani et al., 1999; Kim et al., 1998; Olivar and Laird, 1999), decrease hypersensitivity following sciatic nerve ligation (Mao et al., 1993), and attenuate tactile-evoked nociception in an animal model of allodynia (Bennett and Crooke, 1994; Yaksh and Hammond, 1982; for review, see Dickenson et al., 1997). The NMDA receptor is a Ca^{2+} permeable ligand-gated ion channel. The receptor exists as a heteromeric assembly of several isoforms of the protein, but the NR1 subunit is essential for channel activity (Monyer et al., 1992). NMDA receptor activation increases intracellular Ca^{2+}, which, in turn, activates a cascade of intracellular events regulating gene expression. The increase in intracellular Ca^{2+} results, among other things, in increased expression of protoonco-

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genes such as c-Fos (Hunt et al., 1987). This immediate early gene is a transcription factor that regulates the activity of other late onset genes and, as such, links short-term synaptic events with the longer-term changes in neuronal function that underlie use-dependent synaptic plasticity.

Noxious peripheral stimulation evokes a rapid, substantial increase in c-Fos expression in spinal nociceptive neurons, with maximal c-Fos immunoreactivity present 2–4 h after formalin injection in L3–5, which are the segments that receive major afferents from the plantar hind paw (Presley et al., 1990). Fos protein increased by noxious peripheral stimulation in spinal neurons is predominantly localized to laminae I/II_0 and V. Furthermore, 25% of lamina I/II neurons and 55% of lamina V neurons that express Fos following noxious peripheral stimulation also colabel for NMDA-R1 (Zhang et al., 1998). This increase of Fos protein immunoreactivity is not just a marker of neuronal activation but seems to be essential for central sensitization because the inhibition of Fos expression by antisense oligonucleotide decreases the second phase of flinching reaction (Hou et al., 1997).

As such, NMDA receptors are thought to be important for the activation of Fos expression and Fos is considered a marker of activity in nociceptive neurons (Hunt et al., 1987; Presley et al., 1990). Because NR1 is the most abundant NMDA receptor subunit in the spinal cord and is essential for NMDA receptor function, we speculated that NR1 participates in the nociception and regulation of pain-induced c-*fos* gene expression. To test this hypothesis, we treated rats with antisense oligodeoxynucleotides to the NMDA-R1 subunit and assessed the nociceptive behavior and, as a biochemical marker of activation of spinal nociceptors, expression of the immediate–early gene c-*fos*.

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats (Harlan), weighing 300–350 g (Charles River Laboratories, Wilmington, MA), were used in this study. Animals were housed in a temperature- and humidity-controlled room with a 12-h light–dark cycle. Food and water were available ad libitum. All animal protocols were approved by the IACUC at Harvard Medical School, Boston, MA.

2.2. Catheter installation

Catheter installation into the subarachnoid space was performed as described elsewhere (Yaksh and Rudy, 1976). Rats were anesthetized with halothane, and a 2-cm midline incision was made over the atlanto–occipital junction. Using blunt dissection, deep layers of muscle were separated from bone, and the atlanto–occipital membrane was cut with a needle until clear CSF flowed freely. A catheter (PE 10) was carefully inserted into the subarachnoid space, and the incision was closed using wound clips. Rats were closely observed after awakening and, if motor abnormalities appeared, were not used for subsequent experiments. In addition, to check catheter position, 5 μl of 2% lidocaine was administered through the catheter; transient, reversible hindlimb weakness was taken as evidence of proper catheter position.

2.3. Oligodeoxynucleotides

Oligodeoxynucleotides were purchased from a commercial supplier (Oligos Etc., Wilsonville, OR) and dissolved in 0.9% saline to a concentration of 0.5 nmol/μl. On the first, third, and fifth postoperative days, the ODN was injected into the subarachnoid space using a Hamilton syringe, in a total volume of 10 μl, followed by 10 μl of saline to flush the catheter. The antisense ODN to NMDA-R1 was a 20-mer targeted to nucleotides −1 to +19 of the NMDA-R1 gene with the sequence 5′-GCA GGT GCA TGG TGC TCA TG-3′ (NM_017010, bp 26–5284). We selected the antisense oligonucleotide against NMDA-R1 that has proven effective and specific in previous studies following intracerebroventricular (Wahlestedt et al., 1993), intrastriatal (Standaert et al., 1996), or intrathecal (Garry et al., 2000; Yukhananov et al., 2002) administration. Control rats received a treatment regimen of either vehicle alone (saline), the corresponding sense ODN (sense ODN sequence=5′-CAT GAG CAC CAT GCA CCT GC-3′), or a missense ODN with four mismatched bases (missense ODN sequence=5′-GCA GG**C TAG** TGG TGC TCA TG-3′). One base at each end of the ODN was phosphothioated to enhance stability. All of the ODNs were searched to exclude the possibility that they matched confounding sequences in the GenBank database. There were 12 rats in the saline and antisense groups and 6 in the sense and missense groups. Throughout the course of treatment, animals were assessed for evidence of toxicity, such as poor grooming, impaired bowel or bladder function, difficulty walking, and inability to stand on the hindlimbs.

2.4. Formalin test

On the sixth postoperative day, nociception was assessed behaviorally with the formalin test and biochemically by c-Fos immunohistochemistry. Formalin was prepared from a 37% formaldehyde solution by 1:19 dilution with 0.9% saline and 100 μl of the 5% formalin solution administered into the plantar surface of the left hind paw with a 27-gauge needle. The rat was then placed in an individual Plexiglas cage with wood shaving and the number of flinches of the injected paw counted over a 60-min period by an observer blinded to the treatment that the animal had received. Flinching was characterized as shaking or lifting of the affected paw; it usually occurs as a sharp motion, with rippling across the back. Flinching typically occurs in two

phases. Phase 1 develops immediately after injection, continues for about 5 min and is followed by a 10- to 15-min quiet period. Phase 2 begins about 20 min after injection and lasts for approximately 40–50 min (Goto et al., 1995). Phase 1 reflects the activation of peripheral nociceptors, whereas phase 2 reflects mainly tonic discharges in spinal nociceptive neurons. Hence, for the formalin test, data are presented as the percent change in flinches as compared with a vehicle-treated control group for phases 1 (0–5 min) and 2 (20–60 min).

2.5. Immunohistochemistry

Spinal c-Fos expression was assessed by immunohistochemistry in a subset of ODN and control rats 2 h after the injection of formalin. For this purpose, rats were deeply anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg, Abbott Laboratories, North Chicago, IL) and transcardially perfused with 150–200 ml of phosphate buffered saline (PBS, pH 7.4), followed without interruption by 4% paraformaldehyde in PBS. The lumbar spinal cord was removed immediately and the right ventral side of the lumbar enlargement area marked by a nick with a needle. The cord was postfixed for 4–8 h in the final perfusate and then transferred to PBS containing 20% sucrose for cryoprotection. The cord was stored overnight at 4 °C, and then 50-μm sections were cut at the level of L4/5 using a sliding microtome. Refrigerated sections were preincubated with 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h and then incubated for 48 h at 4 °C with primary c-Fos antibody (Oncogene Research Products, Cambridge, MA) at a concentration of 1:500 in PBS/0.3% triton X-100 (Sigma, St. Louis, MO)/2% goat serum/and 0.1% sodium azide. The primary antibody was removed by three washes with 0.5 M PBS/0.3% triton X-100/1% goat serum, and sections were incubated for 2 h at room temperature in 0.5% biotinylated anti-Rabbit IgG (Vector Laboratories) and 1.5% goat serum in 0.5 M PBS/0.3% triton X-100. After three washes in 0.5 M PBS/0.3% triton X-100, sections were incubated for 2 h at room temperature with freshly prepared horseradish peroxidase avidin–biotin complex (Vector Laboratories) diluted in PBS/0.3% triton X-100. Following one wash with 0.5 M PBS/0.3% triton X-100 and two with 50 mM Tris (pH 7.6), the sections were incubated for 10 min at room temperature in 0.5% 3,3′-diaminobenzidine (DAB, 10 mg/tablet, Sigma)/50 mM Tris/0.4% NH_4Cl/20% beta-D-glucose/1% nickel ammonium sulfate solution. Sections were then transferred into 0.5% DAB solution/0.08% glucose oxidase for 1–5 min until dark blue. Sections were finally washed several times with 50 mM Tris and transferred to subbed slides and were allowed to air dry. Slides were dehydrated in graded ethyl alcohol (70%, 95%, and 100%) for 5 min each and treated with 1:1 chloroform/100% ethyl alcohol for 30 min, incubated in xylene, and cover slipped. Dark blue stained cells were counted manually under a light microscope in the lumbar 4–5 region of the spinal cord by an observer blinded to the treatment group. Measurements were made ipsilateral and contralateral to formalin injection in the laminae I/II and V from at least four randomly chosen sections from each animal.

2.6. Statistical analysis

The statistical analysis of the behavioral data was performed with one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons, for phases 1 and 2. For the c-Fos data, side-to-side comparisons were performed within groups with a paired *t*-test, and analysis of differences between groups was performed with one-way analysis of variance (ANOVA), followed by Duncan's test. Calculations were performed with a statistical software package (SigmaStat 1.0, Jandel Scientific, San Francisco, CA).

3. Results

There were no untoward effects of ODN administration. Treated rats were clinically indistinguishable from controls with respect to general appearance, ambulation, tolerance to handling, etc. Although we did not formally assess motor or bowel and bladder functions, there was no gross evidence of impairment in these functions. Formalin injection produced the typical biphasic behavioral response (Goto et al., 1995; Yukhananov et al., 2002). In vehicle (saline)-treated rats, flinching began immediately following formalin injection

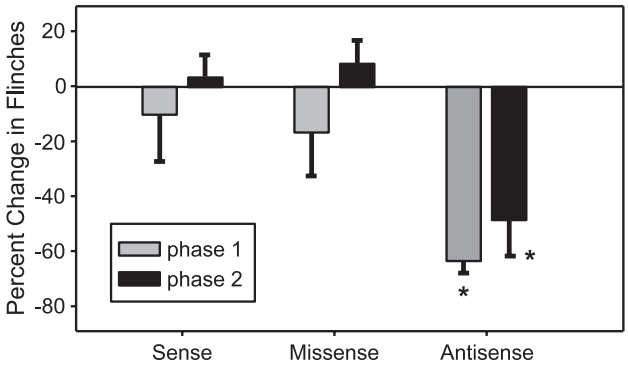

Fig. 1. Effect of treatment with antisense ODN and the corresponding sense and missense ODNs on formalin-evoked flinching behavior. Data were divided into phases 1 (0–5min; gray bar) and 2 (20–60 min; black bar). Rats received three subarachnoid injections of saline or 5 nmol oligodeoxynucleotide over a 5-day period, as described in the text, and formalin testing was performed on day 6. Data are expressed as mean±S.E.M. percent change compared with the saline control. *$p<0.05$ compared with the saline, sense, and missense groups. There were 12 rats in the saline and antisense groups and 6 in the sense and missense groups. Compared with the saline control, there was no effect of the sense or missense ODN, whereas antisense ODN reduced flinching during both phases 1 (37% of control) and 2 (53% of control). The baseline for phase 1 corresponds to 42 flinches; for phase 2, the baseline is 132 flinches.

(phase 1), subsided for 5–10 min, and then recurred for up to 40–50 min (phase 2).

Treatment with the sense or missense NR1 ODNs did not alter behavior during either the phase 1 or 2 of the formalin test (Fig. 1). In contrast, treatment with antisense ODN markedly decreased flinching during phases 1 and 2 (Fig. 1).

The c-Fos data are consistent with the behavioral results. As anticipated, formalin injection produced a substantial increase in Fos-like immunoreactivity (Fos-IR), predominantly in the laminae I/II$_0$ and V of the lumbar enlargement ipsilateral to injection in comparison with the contralateral side (Fig. 2). This formalin-induced expression of Fos-IR was not altered by treatment with the sense ODN (Table 1). However, in rats pretreated with antisense ODN, formalin-induced Fos-IR expression was significantly decreased (Table 1, Fig. 2).

Table 1
Effect of antisense ODN on formalin-evoked c-Fos-like immunoreactivity in the lumbar spinal cord

	Lamina I/II$_0$	Lamina V
	Ipsilateral/contralateral	Ipsilateral/contralateral
Saline (12)	23±4/2±1	30±5/4±1
Sense (6)	16±3/2±1	27±4/5±2
Missense (6)	19±4/1±1	20±3/3±1
Antisense (12)	8±1*/2±1	11±2*/2±1

Rats received three subarachnoid injections of saline or 5 nmol of oligodeoxynucleotide over a 5-day period, as described in the text. On day 6, lumbar cord was removed 2 h after formalin injection. Data are expressed as mean±S.E.M. number of positive neurons in the L4/5 segment for the number of animals in parentheses.

* Compared with the corresponding side in saline control rats, $p<0.05$.

4. Discussion

This study demonstrates that subarachnoid administration of an antisense oligodeoxynucleotide targeted to the NMDA-R1 subunit elicits inhibition of formalin-induced nociceptive behaviors and Fos-IR. Antisense ODN, which targets the area around the translation initiation codon of NR1, was effective in this regard. Moreover, this action appears to be sequence specific because no effects were observed after treatment with the corresponding sense or missense oligodeoxynucleotides. We did not formally assess neurologic function, but this antinociceptive effect occurred without other obvious functional impairment, such as a motor deficit or incontinence. Although NMDA receptor antagonists can produce motor dysfunction, the absence of motor impairment is in concordance with previous studies using NR1 antisense probes (Garry et al., 2000; Rydh-Rinder et al., 2001) and is interesting because R1 is abundantly expressed in the ventral portion of the spinal cord (Tolle et al., 1993).

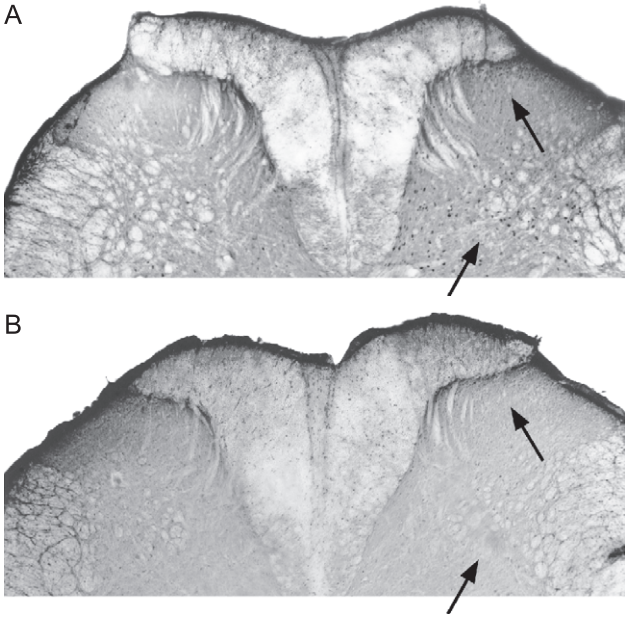

Fig. 2. Effect of treatment with antisense ODN on c-Fos immunoreactivity in spinal cord. Rats received three subarachnoid injections of saline (A) or 5 nmol of antisense ODN (B) over a 5-day period, as described in the text. On day 6, lumbar cord was removed 2 h after formalin injection and stained as described. There is a marked increase of c-Fos-labeled cells at the ipsilateral side following formalin injection after saline (Panel A, arrows indicate c-Fos staining) but not antisense ODN pretreatment (Panel B, arrows show the absence of c-Fos staining in the same areas of the spinal cord). The apparent black staining of dorsal columns is blue background on slides.

That NMDA receptors and c-Fos play a role in nociception has long been known. Competitive and non-competitive NMDA receptor antagonists inhibit the response of dorsal horn nociceptive neurons to noxious stimulation and attenuate hyperalgesic behaviors in a variety of models (Dickenson et al., 1997). Similarly, spinal c-Fos expression following noxious stimulation has been shown to correlate with behavioral hyperalgesia (Dubner and Ruda, 1992). The Fos protein is rapidly and transiently expressed following noxious peripheral stimulation, and drugs such as opiates and NMDA receptor antagonists block both noxious stimulation-induced Fos expression and behavioral hyperalgesia. This is important because the Fos protein, which forms a heterodimer with members of the jun family, is a transcription factor that controls the activity and expression of later response genes. As such, Fos may mediate long-term changes in synaptic plasticity that contributes to hyperalgesia. Indeed, the fact that the intrathecal administration of an antisense oligodeoxynucleotide to c-*fos* produces parallel suppression of c-Fos, and behavioral hyperalgesia suggests a causal relationship between the two events (Hou et al., 1997).

This study extends these observations and suggests that NR1 participates in both behavioral hyperalgesia and c-*fos* expression in the formalin model. The NMDA receptor is an ion channel heteromeric complex of NMDA-R1 and R2 A–D subunit families. The NMDA-R1 subunit is essential for forming a functional receptor (Monyer et al., 1994). In the spinal cord, NMDA-R1 is highly expressed in the dorsal and

ventral regions (Tolle et al., 1993); the various NR2 isoforms are significantly less abundant (Yukhananov et al., 2002). Because of this receptor complexity, antisense oligonucleotides have been used to study the role of the R1 subunit in nociception. In one study (Kolhekar et al., 1997), an antisense oligonucleotide against the R1 subunit of the NMDA receptor attenuated thalamic neuropathic pain. Similarly, an antisense to NMDA-R1 is reported to decrease pain behavior both following intrathecal administration of glutamate and in the formalin test in rats and mice (Garry et al., 2000; Rydh-Rinder et al., 2001). Our data are consistent with these results. An antisense ODN against NR1 reduced phases 2 and 1 behavior about 50% and decreased the number of Fos positive cells ipsilateral to stimulation in the laminae I/II and V by approximately 60%, whereas neither the corresponding sense nor missense ODNs had any effect. Furthermore, our results are consistent with the action of NMDA receptor antagonists. The administration of competitive and noncompetitive NMDA receptor antagonists before formalin injection markedly attenuates phase 2 behavior; phase 1 behavior is also reduced, but to a lesser degree (Hunt et al., 1987; Vaccarino et al., 1993; Millan and Seguin, 1994; Chaplan et al., 1997; Haley et al., 1990). This difference in the effect on phase 1 compared with our results might be explained by pharmacodynamic differences inasmuch as antisense treatment continued for several days, whereas antagonists are usually injected just prior to formalin. Alternatively, differences could be related to variable contributions from spinal and supraspinal sites of action such that intrathecally administered oligos may have fewer supraspinal effects than do either spinally or systemically administered NMDA antagonists. This is relevant because supraspinal NMDA receptor blockade influences the formalin response (McKenna and Melzack, 2001). Nonetheless, the overall similarity between the actions of noncompetitive NMDA receptor antagonists and the antisense ODN provides additional evidence that NR1 is important for Fos expression and, correspondingly, noxious stimulation-induced synaptic plasticity.

Our treatment regimen was chosen based on results from this and other laboratories. For example, a single, intraparenchymal injection of an unmodified NMDA-R1 antisense (15 nmol) into the neostriatum proved sufficient to affect the behavior and decrease the expression of mRNA and protein (Lai et al., 2000). In addition, using this oligodeoxynucleotide and treatment regimen, we have shown that NR1 mRNA is reduced about 30%, without a coincident change in mRNA, for closely related subunits (Yukhananov et al., 2002). This is not to say that a longer treatment interval or more frequent dosing might not have produced a greater effect. In a study of μ-opioid receptors using antisense oligonucleotides, a 5-day treatment interval was more effective than a 3-day treatment was (Standifer et al., 1994). Indeed, studies on the turnover of NMDA-R1 in cultured cerebellar granule cells indicate that NR1 exists in two populations, with half-lives of 2 and 34 h (Huh and Wenthold, 1999). This could explain the incomplete effect of our treatment regimen on hyperalgesia and Fos. It is notable, however, that previously described depression in NMDA-R1 mediated synaptic responses in hippocampal brain slices (Soltesz et al., 1994), receptor binding (Rydh-Rinder et al., 2001), and aversive behavior, after formalin injection (Garry et al., 2000) following 2–2.5 days of treatment with an unmodified NR1 antisense, is on the order of 30%. This is similar in magnitude to the effects we observed with the NR1 antisense compound. Although we did not measure NR1 protein or mRNA in this study, previous work from several laboratories indicates that the NR1 antisense used here is specific and effective in terms of reducing receptor binding and protein expression. (Garry et al., 2000; Lai et al., 2000; Rydh-Rinder et al., 2001; Standaert et al., 1996; Wahlestedt et al., 1993; Yukhananov et al., 2002). Thus, although our treatment interval was brief, there is ample reason to believe that it was adequate to decrease the expression of the targeted mRNA.

One of the main issues surrounding the use of antisense oligonucleotides concerns appropriate controls. Antisense oligonucleotides can produce non- or sequence-specific effects that occur independently of an interaction with messenger RNA. Thus, a sense or missense compound is a necessary control, although these too may produce non-specific effects. In this study, the control oligodeoxynucleotides shared several properties with the corresponding antisense ODN, including GC content and secondary structure, but nevertheless had no effect in either the behavioral or immunohistochemical assay. In addition, in previous work, the sequence specificity of the antisense ODN effect was confirmed by the selective decrease of the appropriate mRNA (Yukhananov et al., 2002). To further minimize potential for biases, however, the behavioral analysis and counting of Fos-IR cells were conducted by a blinded observer.

In summary, an NR1 antisense ODN inhibits nociceptive behavior and spinal Fos protein expression in the formalin model in rats. This supports the concept that the NR1 subunit of the NMDA receptor plays an important role in the development of noxious stimulation-induced synaptic plasticity and spinal Fos expression in this model.

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