An Antisense Oligonucleotide to the \( N \)-Methyl-\( D \)-aspartate (NMDA) Subunit NMDAR1 Attenuates NMDA-Induced Nociception, Hyperalgesia, and Morphine Tolerance

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ABSTRACT

We determined whether the i.t. administration of an 18-mer phosphodiester antisense oligodeoxynucleotide (ODN) that reduces the expression of the rat NMDAR1 subunit of the \( N \)-methyl-\( D \)-aspartate (NMDA) receptor would affect nociceptive behaviors and prevent the development of morphine tolerance. Rats received 5 \( \mu \)l of i.t. saline, 30 nM antisense, or mismatch ODN twice a day for 5 days (NMDA-induced nociception, NMDA-induced thermal hyperalgesia, NR1 mRNA, and ligand binding studies) or for 3 days (formalin study). For the tolerance study, 5 days of ODNs or saline were followed by 3 days of concurrent administration of ODNs or saline (twice a day) and i.t. morphine (three times a day). Antisense, but not mismatch, results in the reduction of formalin phase 2 flinching by 50%, the spinal cord dorsal horn levels of NMDAR1 mRNA by 30%, and ligand binding by 50%. The i.t. \( ED_{50} \) for NMDA-induced nociceptive behaviors is doubled, and thermal hyperalgesia is blocked by antisense treatment. The effects of antisense on NMDA-induced nociception and thermal hyperalgesia are completely reversed by discontinuing antisense. The coadministration of antisense with increasing doses of i.t. morphine for 3 days attenuates the development of morphine tolerance. These results demonstrate that an in vivo antisense targeting of the NMDAR1 subunit results in antihyperalgesic effects and a partial blockade of spinal morphine tolerance. They provide additional support for the critical role of the NMDA receptor in these forms of spinal nociception and in the development of morphine tolerance and suggest the potential therapeutic utility of this approach.

Recent studies have provided compelling evidence that \( N \)-methyl-\( D \)-aspartate (NMDA) receptors are involved in the development of morphine tolerance and in certain hyperalgesia states (Inturrisi, 1997; Mao, 1999, 2002). Following the original observation that MK801 ([+]-5 methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate) can prevent the development of morphine tolerance by Trujillo and Akil (1991), many studies have demonstrated the ability of an NMDA receptor antagonist, acting at a number of sites on this receptor-ion channel complex, to attenuate or reverse the development of tolerance to morphine’s antinociceptive (analgesic) effects (Inturrisi, 1997; Mao, 1999).

Intense or noxious stimuli that are associated with tissue injury involve NMDA receptor-mediated neuroplasticity in the spinal cord dorsal horn, a process described as central sensitization (Woolf and Costigan, 1999). Central sensitization results in increased synaptic efficacy, and the recruitment of normally subliminal inputs leads to the spread of pain sensitivity beyond the site of injury (secondary hyperalgesia) and the generation of pain in response to low-threshold inputs (allodynia) (Woolf and Salter, 2000). The hypersensitivity response to intraplantar formalin involves both peripheral and central sensitization (for references, see South et al., 2003). A conditional deletion of the NR1 subunit of the NMDA receptor has demonstrated the essential contribution of the NMDA receptors located on lumbar spinal cord dorsal horn neurons to central sensitization and injury-related behaviors. An increase in the number of sites on this receptor-ion channel complex, to

Abbreviations: NMDA, \( N \)-methyl-\( D \)-aspartate; ODN, oligodeoxynucleotide; CDR, cumulative dose response; TPW, thermal paw withdrawal; CNS, central nervous system; SpC, spinal cord dorsal horn; PAG, periaqueductal gray; TCA, trichloroacetic acid; MDL 105,519, 3-(2-phenyl-2-carboxyethenyl)-4,8-dichloro-1H-indole-2-carboxylic acid; CI, confidence interval; BL, baseline; PKC, protein kinase C.
induced pain (South et al., 2003). Intrathecal administration of antisense to the NR1 subunit of the NMDA receptor results in a reduction in formalin-induced nociceptive behaviors during phase 2, the part of the response associated with central sensitization (Garry et al., 2000; Rydh-Rinder et al., 2001; Yukhananov et al., 2002).

We observed that D-methadone, a compound with NMDA receptor antagonist activity in binding and functional assays, can attenuate morphine tolerance, thermal hyperalgesia, and the nociceptive behaviors produced by i.t. NMDA and phase 2 of the formalin response at similar doses (Shimoyama et al., 1997b; Davis and Inturrisi, 1999). Taken together, these observations suggest a common site of action for the initiation of these responses at NMDA receptors in the spinal cord dorsal horn. A prediction from the studies cited above is that NR1 antisense treatment should affect not only phase two of the formalin response but also spinally mediated morphine tolerance and i.t. NMDA-induced thermal hyperalgesia and nociceptive behaviors.

The present study examined this hypothesis by measuring the ability of the same i.t. dosage of an 18-mer phosphodiester antisense oligodeoxynucleotide (ODN) directed against rat NMDAR1 subunit to affect the development of spinal morphine tolerance and i.t. NMDA-induced pain behaviors in the rat.

Materials and Methods

Each study was approved by the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

Intrathecal Catheterization. To facilitate the administration of the ODNs, morphine, and NMDA, a catheter was placed in the i.t. space. Under halothane anesthesia, a PE-10 tube was inserted through a small hole made in the atlanto-occipital membrane and threaded 9 cm down the i.t. space to the lumbosacral level of the spinal cord of adult male Sprague-Dawley rats weighing 300 to 375 g (Shimoyama et al., 1997b). Each i.t. injection was delivered in a volume of 5 μl, followed by a 10-μl saline flush. A cannulated rat with any signs of paralysis was excluded from the study. Treatment groups averaged 10 animals. The animals were observed after cannulation and after each treatment for signs of loss of motor coordination.

ODNs and Drugs. The NMDAR1 antisense is an 18-mer phosphodiester ODN (‘5’ GAGCAGGCTCACGGCTGCT 3’) targeted to the nucleotide sequence 4 through 21 that directly follows the initiation codon of rat NMDAR1. The mismatch ODN (‘5’ GAGCAGGCTGCTGCTGCT 3’) contains the same C:G nucleotide content as the antisense but in a different sequence (Wahlestedt et al., 1993). A database search using the BLAST program (National Center for Biotechnology Information, Bethesda, MD) indicated that the antisense sequence was specific for rodent NMDAR1, and the search did not identify a corresponding rodent sequence for the mismatch. The ODNs were obtained from Midland Certified Reagent (Midland, TX). The morphine sulfate was obtained from Mallinckrodt (St. Louis, MO) and NMDA from Sigma/RI (Natick, MA). The compounds were dissolved in 0.9% sterile saline, and the pH of the final NMDA solution was adjusted to 7.0 with 1 N NaOH.

ODN Administration. Figure 1 presents a timeline of the ODN or saline treatment schedules. For the NMDA-induced nociception, NMDA-induced thermal hyperalgesia, NR1 mRNA, and ligand binding studies, the ODN treatment on days 1 through 5 was twice a day (9:00 AM and 5:00 PM), given by the i.t. route (Fig. 1A). The groups received 5 μl of saline or the same volume containing 30 nM NR1 of antisense ODN or 30 nM of mismatch ODN. For the formalin study, the ODN treatment was twice a day for 3 days.

NMDA-Induced Nociceptive Behavior. Intrathecal NMDA produces a short-lasting behavioral response, which consists of an intense caudally directed biting, licking, and scratching behavior (Shimoyama et al., 1997b). Doses of NMDA from 0.68 to 5.4 nM per rat were administered i.t. using a 3-min interinjection interval. A responder was defined as a rat in which NMDA produced scratching, biting, and licking of the caudal dermatomes that was at least 30 s in duration. The ED50 value was estimated using cumulative dose-response (CDR) analysis as described below for morphine. To determine whether the effects of the antisense treatment were reversible, no additional ODN or saline treatments were administered to these animals, and on day 13, the ED50 for i.t. NMDA was estimated (Fig. 1A).

NMDA-Induced Hyperalgesia. In a separate group of animals, the change in thermal paw withdrawal (TPW) latency (hyperalgesia) following i.t. NMDA was determined (Davis and Inturrisi, 1999). In the TPW test, a rat is placed on an elevated glass surface covered by an inverted plastic cage. A radiant heat source is focused on the plantar surface of the hind paw, and the time until the rat withdraws its hind paw is automatically determined. A cutoff time of 30 s is imposed to avoid tissue damage, and the intensity of the heat source is adjusted so that baseline latencies are between 10 and 15 s. The testing was done on each hind paw, and these results were averaged for each time point. A previous study found that an i.t. dose of NMDA of 7.8 nM per rat produced a peak decrease in the mean TPW latency of approximately 8 s where the predrug TPW latency averaged 12 s (Davis and Inturrisi, 1999). TPW latency was measured at baseline and 5 min before the i.t. administration of NMDA (time 0) and then every 5 min for a total of 30 min. On day 0 (before ODN treatment), the baseline and the response to i.t. NMDA of each animal was determined. Next, half the animals were randomly assigned to receive antisense for 5 days; the other half received mismatch ODN treatment. Baseline and post-i.t. NMDA testing was repeated on day 6. To determine whether the effects of the antisense treatment were reversible, no additional ODN or saline treatments were administered to these animals, and on day 13 baseline and post-i.t. NMDA testing was repeated (Fig. 1A).
The Formalin Test. A 50-μl volume of 5% formalin was injected into the plantar surface of the right hind paw, and immediately following the injection, the rat was placed in a test chamber and was observed by an individual blinded as to the treatment. The total numbers of flinches, defined as quick shakes of the injected hind paw, were recorded for the next 60 min. The formalin injection resulted in a biphasic time course of flinching behaviors (phase 1, 0–10 min, phase 2, 10–60 min; Shimoyama et al., 1997a).

Tail-Flick Test and Cumulative Dose-Response Analysis. To assess the algogenic properties of morphine, the tail-flick was used. The tail-flick apparatus (EMD, Richmond, VA) emits radiant heat to the tail, and the time from the onset of the heat stimulus to the withdrawal of the tail (tail-flick latency) was measured. The intensity of the radiant heat was adjusted so that the baseline latencies were between 2.5 and 3.5 s. To avoid causing tissue damage, the heat stimulus automatically turned off at 10 s (cut-off latency). The mean tail-flick latency was calculated from two repeated measurements. Baseline latency was obtained before drug administration. Subsequent response latencies were determined at the time of peak analgesia, which was 10 min after i.t. morphine.

After measuring the baseline tail-flick latencies, increasing doses of morphine were administered until each animal responded to the analgesic (CDR) (Elliott et al., 1994a,b). Cumulative dose-response analysis allows a reproducible estimate of the ED50 value to be obtained with fewer animals than are required with single-dose analysis (Elliott et al., 1994a,b). A quantal analgesic responder was defined as one whose mean tail-flick latency response was at least twice the value of the mean baseline latency (Shimoyama et al., 1996). The percentage of quantal responders in each group at each cumulative dose was calculated, and a cumulative dose-response curve was constructed. The changes in the median effective dose (ED50) of morphine determined from the curves were used to express the changes in the relative potency of morphine (Elliott et al., 1994b).

Antisense Treatment and Responsiveness to Morphine. To evaluate the effects of antisense treatment on the antinociceptive response to morphine, animals were treated with i.t. ODN or saline twice a day for 5 days as described above and see Fig. 1B. On day 6, a morphine CDR was conducted to determine whether ODN treatment affected the response (ED50 value) to morphine. Then, we began an escalating schedule of i.t. morphine that has been shown to produce tolerance (Shimoyama et al., 1996). This schedule included two doses of 10 μg/rat of i.t. morphine on day 6, three doses of 20 μg/rat on day 7, and three doses of 40 μg/rat on day 8 (Fig. 1B). The twice daily saline or ODN treatment was continued on days 6 to 8, and on day 9, only the AM dose of ODN or saline was administered (Fig. 1B). On day 10, the morphine CDR was repeated.

Quantitation of NMDAR1 mRNA: Tissue Collection and RNA Extraction. NMDAR1 (NR1) mRNA levels were determined using RNA extracts of CNS tissues obtained from rats treated as described above for 5 days with i.t. saline or ODN (Fig. 1A) were removed and frozen. In a cryostat, 6-μm tissue sections of the lumbar spinal cord were thaw-mounted onto gelatin-subbed microscope slides and refrozen for later use (within 48 h).

Glycine is a coagonist for the NMDA receptor. The glycine binding site is located on the NR1 subunit of the NMDA receptor (Dingledine et al., 1999), the target for our antisense. Under the conditions of our binding assay, [3H]glycine and the glycine site antagonist [3H]MDL 105,519 (3-[2-(phenyl-2-carboxyethyl)-4,6-dichloro-1H-indole-2-carboxylic acid] label the glycine recognition site on the NR1 subunit of the NMDA receptor (Baron et al., 1996). For the [3H]glycine autoradiography, slides were washed for 20 min in ice-cold 50 mM Tris acetate buffer, pH 7.4, preincubated twice at 30°C for 10 min, and then incubated for 20 min in ice-cold buffer containing 100 mM [3H]glycine to assess total binding. NMDAR1 subunits were selectively labeled by the inclusion of 10 μM glutamate and 100 μM strychnine, which displace all detectable non-NMDAR1 subunit binding. Adjacent sections were incubated under the same conditions in a solution containing radiolabeled ligand in the presence of 100 μM glycine and 100 μM strychnine to assess nonspecific binding. Slides were then washed for 20 s, air dried, and placed against tritium-sensitive film (Hyperfilm-3H, Amersham Biosciences Inc., Piscataway, NJ) with tritium standards for 5 weeks (Microscales; Amersham Biosciences Inc.). For the [3H]MDL 105,519 autoradiography, slides were washed for 20 min in ice-cold 50 mM Tris acetate buffer, pH 7.0, preincubated two sequential times at 30°C for 10 min, and then incubated for 30 min in ice-cold buffer containing 4 mM [3H]MDL 105,519 to assess total binding. Adjacent sections were incubated under the same conditions in a solution containing radio-
labeled ligand in the presence of 1 mM glycine to assess nonspecific binding (Monaghan, 1993). Slides were then washed for 1 min, air dried, and placed against tritium-sensitive film (Hyperfilm-\(^{3}\)H; Amersham Biosciences Inc.) with tritium standards for 4 weeks (Microscales; Amersham Biosciences Inc.).

For image analysis, the films were developed by using standard Kodak developer (D-19) and Kodak Rapid Fix fixative. Radioligand binding levels were determined by computer-assisted image analysis of the autoradiograms (MCID; Imaging Research, St. Catherine's, ON, Canada). Each slice contained sections from the three treatment groups, saline, antisense and mismatch. To reduce the variability in specific binding from slide to slide (interslide variability), we normalized the percentage of specific binding of antisense and mismatch to the saline group on each slide. In previous experiments (Monaghan and Beaton, 1991), we have observed that normalizing values in this manner reduced variability by 74%. This method of analysis takes advantage of the identical experimental conditions that exist for tissue on the same slide.

**Data Analysis.** The quantal morphine dose-response data were analyzed using the BLISS-21 computer program (Shimoyama et al., 1996). This program provides ED\(_{50}\) values, the 95% confidence interval (CI), and relative potency estimates. The remaining data were analyzed by a one-way analysis of variance with subsequent comparisons made using the Student-Newman Keuls test for multiple treatments. The level of significance was \(p < 0.05\).

**Results**

Figure 2 presents a profile of the levels of NMDAR1 (NR1) mRNA obtained by solution hybridization of RNA extracts from selected CNS regions of the rats treated i.t. for 5 days (see Fig. 1A for time line) with saline, antisense, or mismatch ODN. Compared with control and mismatch, the antisense produced a significant bilateral reduction in NR1 mRNA in the SpC that averaged 30% and reduction in the PAG that averaged 9%. Antisense treatment did not result in a significant change in NR1 mRNA in any of the other CNS regions sampled.

Figure 3 compares the binding of two radioligands of \([^{3}\text{H}]\)glycine and \([^{3}\text{H}]\)MDL 105,519 with the lumbar spinal cord of rats treated for 5 days with i.t. saline, antisense, or mismatch ODN. The binding of both ligands, measured under conditions where the binding is relatively specific for the NR1 receptor (see above), was significantly reduced an average 42% for \([^{3}\text{H}]\)glycine and 61% for \([^{3}\text{H}]\)MDL 105,519 by anti-sense, but not by mismatch, ODN treatment, compared with the saline control. Since the values for the \([^{3}\text{H}]\)glycine and \([^{3}\text{H}]\)MDL 105,519 binding after antisense treatment were not significantly different, the overall decrease in NR1 receptor binding averaged 50%.

Table 1 shows that the i.t. NMDA ED\(_{50}\) to produce nociceptive behaviors is increased by 5 days of antisense but not mismatch ODN treatment. NMDA is approximately half as potent in rats treated with NR1 antisense compared with animals treated with mismatch or saline. This antinociceptive effect of the NR1 antisense ODN is completely reversed when the treatment is discontinued on day 6, and the animals are challenged with NMDA on day 13 (see Fig. 1A for time line).

Figure 4 demonstrates that after 5 days of antisense but not mismatch ODN treatment, the decrease in thermal paw withdrawal latency at 5 and 10 min after i.t. NMDA (thermal hyperalgesia) is completely prevented. This antihyperalgesic effect of the antisense ODN is lost when the treatment is discontinued on day 6, and the animals are challenged with i.t. NMDA on day 13 (Fig. 4; see Fig. 1A for time line). The baseline (BL) values were not significantly different as a function of treatment or day (Fig. 4).

Figure 5 shows that after 3 days of antisense but not mismatch ODN treatment, formalin-induced flinching behavior during phase 2 is reduced an average of 48% compared with saline or mismatch. Phase 1 flinching was not affected by the ODN treatment (Fig. 5).

**TABLE 1**

NMDAR1 antisense treatment attenuates NMDA-induced nociceptive behaviors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>NMDA ED(_{50}) ((\mu g/rat))</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6</td>
<td>292</td>
<td>231–369</td>
</tr>
<tr>
<td>Antisense</td>
<td>6</td>
<td>555*</td>
<td>432–721</td>
</tr>
<tr>
<td>Mismatch</td>
<td>6</td>
<td>237</td>
<td>180–305</td>
</tr>
<tr>
<td>Saline</td>
<td>13</td>
<td>324</td>
<td>259–403</td>
</tr>
<tr>
<td>Antisense</td>
<td>13</td>
<td>321</td>
<td>250–411</td>
</tr>
<tr>
<td>Mismatch</td>
<td>13</td>
<td>289</td>
<td>223–371</td>
</tr>
</tbody>
</table>

* Significantly different (\(p < 0.05\)) from each of the other treatment groups.
these animals. To accomplish this, three of the groups (saline morphine, antisense morphine, and mismatch morphine) received repeated doses of i.t. morphine (Table 2; Fig. 1 B). Four groups, including a group that had received only saline treatment (saline + saline), were then evaluated for morphine sensitivity using the morphine CDR on day 10 (Table 2; Fig. 1 B). The three times per day i.t. morphine dosing schedule resulted in a 25-fold increase in the i.t. morphine ED_{50} value and a corresponding decrease in the relative potency of i.t. morphine (saline + morphine group on day 10 in Table 2). The morphine ED_{50} value was increased approximately 6 fold in the antisense + morphine group on day 10, compared with the saline + saline group (Table 2), which was designated the control for relative potency comparisons. In contrast, the increase in the morphine ED_{50} value of 18 fold in the mismatch + morphine group was not significantly different from the value of the saline + morphine group on day 10 (Table 2). Thus, the development of morphine tolerance was significantly less in the antisense but not in the mismatch-treated group (Table 2).

Motor coordination was assessed after 3, 5, or 8 days of ODN or saline treatment by determining the ability of each animal to negotiate a 60° vertical mesh. None of the animals receiving ODN treatment showed a reduction in response compared with the saline-treated controls.

**Discussion**

Antisense-based approaches have been used to evaluate the in vivo contributions of many proteins involved in pain behaviors (Stone and Vulchanova, 2003). The NR1 antisense ODN used in this study has also been used successfully in vivo to attenuate other NMDA receptor-mediated events, including withdrawal from morphine, focal brain ischemia, traumatic brain injury, and long-term potentiation in the hippocampus (Wahlestedt et al., 1993; Matthies et al., 1995; Sun and Faden, 1995; Zhu and Ho, 1998). When delivered into the cerebrospinal fluid at doses from 15 to 30 nM per rat, the unmodified ODNs such as the NR1 antisense are taken up by neurons and appear to be remarkably stable and nontoxic (Wahlestedt, 1994). The stability of unmodified ODNs in cerebrospinal fluid is due at least in part to the low levels of nucleases in this biofluid (Wahlestedt, 1994). Because the antisense ODN is charged, repeated doses or continuous infusion should favor some diffusion from the site of adminis-

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>Morphine ED_{50} (μg/rat)</th>
<th>95% CI</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline + saline</td>
<td>6</td>
<td>1.3</td>
<td>0.7–2.5</td>
<td>1.00</td>
</tr>
<tr>
<td>Saline + morphine</td>
<td>6</td>
<td>1.1</td>
<td>0.7–1.5</td>
<td>1.18</td>
</tr>
<tr>
<td>Antisense + morphine</td>
<td>6</td>
<td>1.6</td>
<td>1.1–2.2</td>
<td>0.81</td>
</tr>
<tr>
<td>Mismatch + morphine</td>
<td>6</td>
<td>1.6</td>
<td>1.1–2.3</td>
<td>0.81</td>
</tr>
<tr>
<td>Saline + saline</td>
<td>10</td>
<td>0.9</td>
<td>0.5–1.9</td>
<td>1.44</td>
</tr>
<tr>
<td>Saline + morphine</td>
<td>10</td>
<td>30.3*</td>
<td>21.7–42.7</td>
<td>0.04</td>
</tr>
<tr>
<td>Antisense + morphine</td>
<td>10</td>
<td>8.1**</td>
<td>5.9–11.0</td>
<td>0.16</td>
</tr>
<tr>
<td>Mismatch + morphine</td>
<td>10</td>
<td>22.7*</td>
<td>16.1–32.1</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Significantly different (p < 0.05) from days 6 and 10 saline + saline groups.

**Significantly different (p < 0.05) from day 10 saline + morphine and mismatch + morphine groups.
tration to supraspinal sites. Our mRNA analysis provides some evidence for limited distribution to the PAG (Fig. 2). However, the effects of this NR1 antisense on mRNA levels were largely confined to the site of administration, the lumbar spinal cord dorsal horn. The duration of antisense treatment of 3 to 5 days was based on previous studies with this ODN (Wahlestedt et al., 1993; Garry et al., 2000; Rydh-Rinder et al., 2001). The turnover rate of hippocampal synaptic NMDAR1 subunits is greater than 48 h (Wahlestedt et al., 1993; Soltesz et al., 1994). We observed that the in vivo blocking activity of the NR1 antisense on nociceptive behavior had disappeared on day 13, which was 7 days after discontinuing the administration of the antisense (Table 1; Fig. 4). Thus, the effects of the antisense on behavior are completely reversible. We found that both NR1 mRNA and NMDA receptor ligand binding in spinal cord were decreased by the NR1 antisense. NR1 mRNA was decreased approximately 30% (Fig. 2) in agreement with the report of Yukhananov et al. (2002). Ligand binding in the spinal cord was decreased an average of 50% (Fig. 3), a reduction that is somewhat greater than the 36 to 44% range found after i.t. administration of this antisense by Rygh-Rinder et al. (2001). We speculate that the NR1 antisense may have acted by inducing translational arrest of NR1 protein synthesis and by forming an NR1 mRNA-DNA duplex that was a substrate for RNase H activity (Wahlestedt, 1994).

In the present study, an antisense knockdown approach was used to obtain in vivo evidence for a role for the NMDA receptor in the lumbar spinal cord dorsal horn in NMDA-induced nociception and in the development of morphine tolerance. We demonstrated the ability of an 18-mer phosphodiester ODN antisense targeted to the rat NMDAR1 subunit (Wahlestedt et al., 1993) to knock down NMDA receptor binding and to affect the expression of nociceptive behaviors produced by NMDA and formalin and the development of morphine tolerance. Previous studies have demonstrated the ability of an antisense knockdown of the NMDA receptor to reduce nociceptive behaviors following intraplantar formalin (Garry et al., 2000; Rydh-Rinder et al., 2001; Yukhananov et al., 2002) and after i.t. NMDA (Garry et al., 2000). We have confirmed and extended these results by demonstrating that an NR1 subunit knockdown has antihyperalgesic effects in a model of i.t. NMDA-induced thermal hyperalgesia (Figs. 4 and 5; Table 1). Native NMDA receptors are hetero-oligomers of subunits that in the rat include the NR1, NR2A through NR2D, and NR3 subunits (Dingledine et al., 1999). In the rat spinal cord dorsal horn, single-cell reverse transcription-polymerase chain reaction has detected the NR1 subunit and each of the four NR2 subunits (Karlsson et al., 2002). Electrophysiological studies of expressed NMDA receptors (Dingledine et al., 1999) and of spinal cord dorsal horn neurons from a conditional deletion of the NR1 subunit (South et al., 2003) demonstrate that this subunit is required to generate characteristic NMDA currents. Thus, a knockdown directed at the NR1 subunit can be expected to reduce the number of functional NMDA receptors. In this regard, the conditional knockout of NR1 that was spatially restricted to the lumbar spinal cord dorsal horn resulted in a greater reduction (73%) in phase 2 formalin-induced nociceptive behaviors (South et al., 2003) than was observed with the NR1 knockdown (Fig. 5). This is consistent with the much more extensive reduction in NR1 mRNA and protein (80 to 90%) in the NR1 knockout than was observed with the NR1 knockdown (Figs. 2 and 3). The immediate response to intraplantar formalin results from the stimulation of primary afferent nociceptors (South et al., 2003), whereas the latter response, phase 2, is generated by a reduced but continuing stimulation of peripheral nociceptors (South et al., 2003) activation of inflammatory mediators and central sensitization triggered by the phase 1 input from the periphery and maintained by the input associated with phase 2 (Woolf and Costigan, 1999). Pharmacological studies using centrally applied receptor antagonists indicate a contribution of NMDA receptors to the behavioral response seen during phase 2 of formalin (for references, see South et al., 2003). The results obtained with the NR1 antisense knockdown together with those obtained in the NR1 KO mouse provide strong convergent evidence that the NMDA receptor is the key player in the pain hypersensitivity expressed during phase 2 of the formalin response. A number of reports have described parallels in the characteristics of nerve injury-induced hypersensitivity and the reduced antinociceptive effectiveness of morphine observed with the development of tolerance (Mao et al., 1995a,b; Mao, 2002). Most importantly, injury-induced hypersensitivity, morphine tolerance, and changes in NR1 mRNA produced by chronic morphine are prevented by blockade of the NMDA receptor (Mao et al., 1995a, 2002; Zhu et al., 2003). Morphine tolerance also involves molecular events at the mu opioid receptor, including posttranslational modifications, internalization, and multimer formation (Nestler and Aghajanian, 1997; Alvarez et al., 2002; He et al., 2002). NMDA receptors do not appear to directly regulate the expression of mu opioid receptors (Tiseo et al., 1994). In the present report, we observed that although an antisense knockdown of NR1 attenuated the development of morphine tolerance, 5 days of NR1 antisense had no effect on the ED₅₀ value for morphine (day 6, Table 2). Thus, the expression of the analgesic response to morphine in the spinal cord dorsal horn does not appear to require normal levels of NMDA receptors, whereas the adaptation to chronic morphine (development of analgesic tolerance) does require this level of NMDA receptor expression. Mu opioid receptor activation results in the activation of protein kinase C (PKC), which can phosphorylate target proteins, including the NMDA receptor, resulting in the removal of the Mg²⁺ blockade of the NMDA ion channel (Chen and Huang, 1992; Mao et al., 1995b). Chronic morphine induces a down-regulation of spinal glutamate transporters resulting in an increase in synaptic glutamate which can provide additional activation of the NMDA receptor complex (Mao et al., 2002). NMDA receptor activation results in the elevation of intracellular levels of Ca²⁺ ion and the activation of intracellular cascades resulting in further activation of PKC and the production of NO (fore references, see Mao, 1999). It has been suggested that PKC and/or NO may modulate mu opioid receptor coupling through G-proteins to K⁺ channels (Mao, 1999; Mao et al., 2002). Our results provide further support for the role of NMDA receptors in both injury-induced pain and morphine tolerance. Furthermore, these results suggest that spinally administered compounds that remain localized, including NMDA receptor antagonists, antisense, or small interfering RNAs targeted to NR1, may yield safer and more useful therapeutic procedures for injury-induced pain and morphine.
tolerance than have been achieved with currently available NMDA receptor antagonists (Chizh et al., 2001).

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References


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