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Functionally differentiating two neuronal nitric oxide synthase isoforms through antisense mapping: Evidence for opposing NO actions on morphine analgesia and tolerance

(MOR-1/opioid/nu receptor)

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ABSTRACT **Several isoforms of neuronal nitric oxide synthase (nNOS) have been identified. Antisense approaches have been developed which can selectively down-regulate nNOS-1, which corresponds to the full-length nNOS originally cloned from the brain, and nNOS-2, a truncated form lacking two exons which is generated by alternative splicing, as demonstrated by decreases in mRNA levels. Antisense treatment also lowers nNOS enzymatic activity. Down-regulation of nNOS-1 prevents the development of morphine tolerance. Whereas morphine analgesia is lost in control and mismatch-treated mice given daily morphine injections for 5 days, mice treated with antisense probes targeting nNOS-1 show no decrease in their morphine sensitivity over the same time period. Conversely, an antisense probe selectively targeting nNOS-2 blocks morphine analgesia, shifting the morphine dose-response curve over 2-fold to the right. Both systems are active at the spinal and the supraspinal levels. An antisense targeting inducible NOS is inactive. Studies with N^G-nitro-L-arginine, which does not distinguish among NOS isoforms, indicate that the facilitating nNOS-2 system predominates at the spinal level while the inhibitory nNOS-1 system is the major supraspinal nNOS system. Thus, antisense mapping distinguishes at the functional level two isoforms of nNOS with opposing actions on morphine actions. The ability to selectively down-regulate splice variants opens many areas in the study of nNOS and other proteins.**

Nitric oxide synthase (NOS) has become increasingly important as our understanding of its diverse biological actions has expanded, especially within the nervous system (1–7). In addition to the documented role of NOS in pain perception (7–10), the NOS inhibitor N^G-nitro-L-arginine (NOArg) also blocks the development of morphine tolerance (11, 12), observations that have now been confirmed and extended to a number of NOS inhibitors (13–19). This blockade of morphine tolerance by NOS inhibitors is consistent with similar actions of a variety of competitive and noncompetitive *N*-methyl-D-aspartate antagonists (15, 20–29).

Three NOS enzymes have been cloned (1–5), but the predominant one in the brain is neuronal NOS (nNOS) (30). A number of nNOS splice variants have been identified, including one lacking exon 2 (31) and others lacking exons 9 and 10 or exon 10 alone (32, 33) (see Fig. 1). The presence of these alternatively spliced isoforms raises the possibility that they may mediate pharmacologically distinct actions. The limited selectivity of traditional NOS inhibitors precludes their use in exploring this question, but antisense paradigms provide an extraordinary selectivity (34–37) and the ability to evaluate the functional activity of splice variants through the selective targeting of individual exons (37–42). In the current study, we have utilized an antisense approach to define the NOS isoforms involved with morphine analgesia and tolerance.

MATERIALS AND METHODS

Morphine sulfate was a gift from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). Halothane was obtained from Halocarbon Laboratory (Hackensack, NJ). [^{3}H]L-arginine was purchased from NEN/Dupont. NOArg was purchased from Sigma. Dowex AG50WX-8-H^+ resin was purchased from Bio-Rad.

Male CD-1 mice (24–32 g; Charles River Breeding Laboratories) were housed in groups of five with food and water *ad libitum*. Animals were maintained on a 12-h light/dark cycle. Compounds were administered intracerebroventricularly (i.c.v.) under light halothane anesthesia as previously reported (43). Response latencies were determined by the radiant heat tailflick assay (44), with baseline latencies between 2 and 3 sec. We used a maximum cutoff score of 10 sec to minimize tissue damage. Antinociception was defined quantally as a doubling or greater of baseline tailflick scores, as previously reported (39, 40, 42). For convenience, the term analgesia is used synonymously with antinociception. Comparisons of single doses were performed using the Fisher Exact Test.

All phosphodiester antisense oligodeoxynucleotides were based upon the mouse sequence (32). Mismatch oligodeoxynucleotides were designed by switching the sequence of two base pairs, keeping the remaining sequence the same. All were purified in our laboratory and dissolved in 0.9% saline before injection (2 μl) (39, 40, 42) as indicated in the figure legends. NOS activity was measured (14, 45). All experiments were performed in triplicate.

Changes in mRNA levels of the nNOS isoforms were determined using reverse transcription–PCR (RT-PCR). Total RNA was extracted from mouse periaqueductal gray (PAG) region using an RNAeasy Mini kit (Qiagen, Chatsworth, CA). The first-strand cDNAs were reverse-transcribed from the total RNA with random hexamers and used in the following PCRs with [α-^{32}P]dCTP. The amount of RNA added was determined by PCR using a set of mouse β_2-microglobulin primers (CLONTECH), yielding an amplified 373-bp fragment. The expression of NOS-1 mRNA was measured by PCR using a sense primer A (5′-CGCAGCTCATCCGCTATGCC-

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Abbreviations: NOS, nitric oxide synthase; NOArg, N^G-nitro-L-arginine; nNOS, neuronal NOS; RT-PCR, reverse transcription–PCR; PAG, periaqueductal gray; i.c.v., intracerebroventricular; i.t., intrathecal.

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3′, nt 1468–1487 of the mouse nNOS; GenBank accession no. D14552) and an antisense primer B (5′-CAATCCACAC-CCAGTCGGCG-3′, corresponding to nt 2057–2076), yielding a 517-bp fragment. The PCR was carried out for 25 cycles, each cycle consisting of a 30-sec melting step at 94°C, a 30-sec annealing step at 63°C, and a 40-sec extension step at 72°C. To assess the expression of NOS-2 mRNA, the double-stranded cDNAs were amplified by the first PCR using the above first-strand cDNAs as template only with the sense primer A for 15 cycles, each cycle consisting of a 1-min melting step at 94°C, a 1-min annealing step at 63°C, and a 1-min extension step at 72°C. The PCR products were then digested with the *Dra*II, which cut twice in the predicted NOS-1 fragments, but not in the NOS-2 fragments. The digested PCR products were used as templates in the second PCR with the sense primer A and the antisense primer B for 35 cycles using the same program as for NOS-1. The amplified fragment size for NOS-2 is 203 bp. All the PCR products were separated in 1.5% thin agarose gel and then exposed to x-ray film. The corresponding bands observed on the film were cut out and counted.

RESULTS

NOS Antisense Actions on mRNA and Enzyme Activity. In view of the limited selectivity of NOArg, we have employed an antisense approach based upon paradigms previously worked out in our laboratory (39–42, 46, 47). We focused upon the full-length nNOS (nNOS-1) and the isoform lacking two exons (nNOS-2), which correspond to exons 9 and 10 in the human homolog (32, 33) (Fig. 1*a*). The levels of nNOS-2 in the brain are quite low, with an abundance less than 10% of that of nNOS-1 and with highest values in the cerebellum and the spinal cord (32). Using RT-PCR, we also confirmed the presence of nNOS-2 in all brain regions examined and its low abundance relative to nNOS-1. Spinal cord levels of nNOS-2 were about 60% of those in the cerebellum, the region with the highest levels (data not shown). Levels in the PAG were only about 40% of those in the cerebellum, while the cortex had the lowest levels (28%). To explore the roles of these isoforms, we designed antisense probes selective for nNOS-1 (antisense B, D and E) or nNOS-2 (antisense C) or probes targeting both (antisense A and F).

First, we examined the efficacy and specificity of the down-regulation of mRNA levels in the periaqueductal gray (Fig. 1 *b* and *c*), a region with high nNOS levels (48) and which is important in opioid analgesia (49). Antisense F, which targets both nNOS isoforms, reduced the levels of nNOS-1 and nNOS-2 mRNA by 48% and 64%, respectively, using an RT-PCR approach to enhance sensitivity. Antisense C, which selectively targets nNOS-2, lowered the levels of nNOS-2 mRNA by 76% without noticeably affecting the levels of nNOS-1. Conversely, antisense D reduced nNOS-1 mRNA levels by 67% without interfering with nNOS-2 mRNA levels. Thus, antisense approaches can selectively down-regulate individual splice variants.

Antisense treatment with a common probe reduced nNOS enzymatic activity. Using the conversion of [^{3}H]arginine to [^{3}H]citrulline to examine NOS activity (14), we observed that antisense F lowered the levels of NOS activity by almost 35% ($P < 0.05$; Fig. 2), a reduction comparable to that seen against the mRNA levels and similar to antisense results in other systems (37, 39, 46). The inactivity of the mismatch control confirms the selectivity of the effect.

NOS Antisense and Morphine Analgesia. First, we examined the time course of antisense A effects following intrathecal (i.t.) administration. As anticipated, intrathecal antisense A adminstration blocked morphine analgesia in a time-dependent manner (Fig. 3). The mismatch probe was inactive, confirming the selectivity of this response. We then examined the relative importance of the two nNOS isoforms at the spinal level using antisense C and D, which selectively target nNOS-2 and nNOS-1, respectively (Fig. 4*a*). Antisense C effectively blocked morphine analgesia ($P < 0.001$) while antisense D was inactive, inferring that nNOS-2 is important in mediating morphine analgesia and nNOS-1 is not. As before, the mismatch probes were inactive.

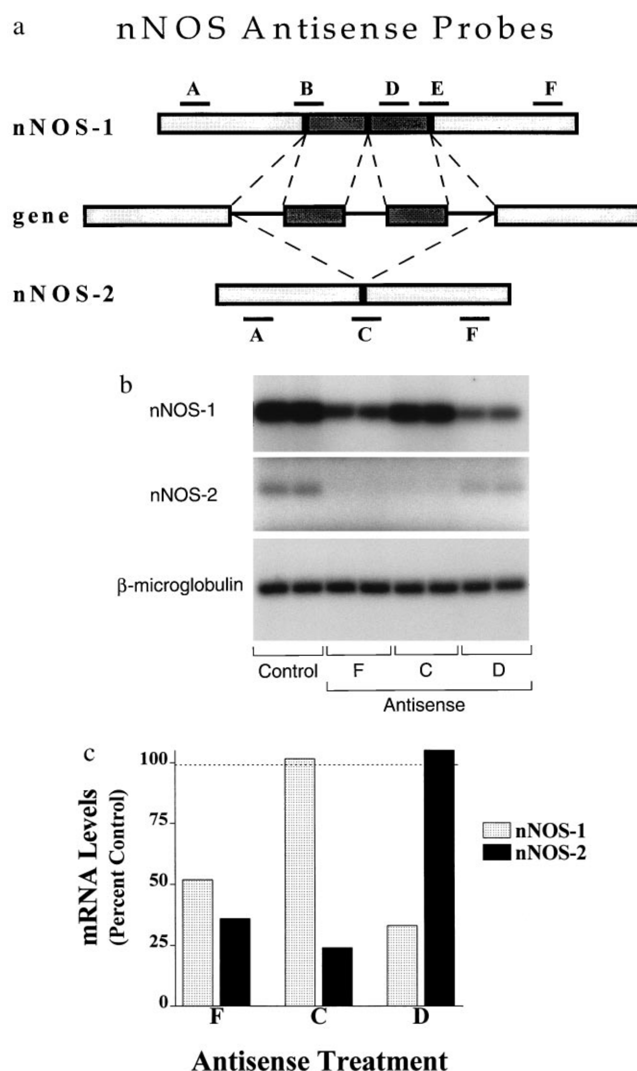

FIG. 1. Effect of antisense probes against nNOS mRNA. (*a*) A schematic of a portion of the nNOS gene is presented which illustrates both nNOS-1 and nNOS-2 as splice variants. Antisense A and F are present on both isoforms. Antisense B and E span the splice regions between the conserved and the additional exons in nNOS-1. Antisense D targets one of the additional exons in nNOS-1. Antisense C spans the splice site where the two exons in nNOS-1 are deleted and is selective for nNOS-2. The sequences of the oligodeoxynucleotides are as follows: antisense A, GGG CTG GGT CAC ACG GAT GG(nt 454–474); antisense B, GCT GTA TAC AGA TCT CTG TGA ACT CC (nt 1595–1621); antisense C, TCT TGG CTA CTT CCT GTG TGA ACT CC (nt 1595–1608, 1922–1935); antisense D, CGA ACG CCA ATC TCC GTG CC (nt 1866–1886); antisense E, TCT TGG CTA CTT CCT CCA GGA TGT TG (nt 1910–1936); antisense F, GAA TCC TCT CCC CGC CCA (nt 2815–2833); mismatch C, TCT GTG CTA CTC TCT CTG TGA ACT CC; mismatch D, CAG ACG CAC ATC CTC GTG CC; mismatch F, GAA TCT CCT CCC GCC CCA (from GenBank accession no. D14552). These correspond to the following exons based upon the human gene: A, exon 2; B, exon 8/9; C, exon 8/11; D, exon 10; E, exon 10/11; F, exon 18. (*b*) The levels of nNOS-1, nNOS-2, and β_2-microglobulin were determined in duplicate control and antisense F treated samples using RT-PCR as described. (*c*) The corresponding bands observed on the film were cut out and counted. The two samples were averaged and plotted as percent of control.

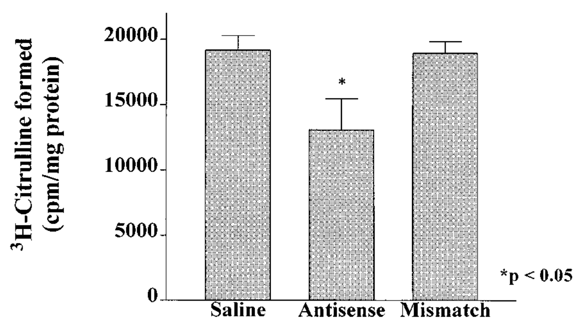

FIG. 2. Effect of antisense treatment on NOS enzymatic activity in the PAG. Groups of mice received antisense F, which targets both nNOS isoforms, or a corresponding mismatch F (20 μg, i.c.v.). The following day the PAG was dissected and pooled to permit determination of nNOS enzymatic activity, measured by the formation of [^{3}H]citrulline from [^{3}H]arginine, as described by Dryer *et al.* (45). Results are the means ± SEM of three independent determinations. The antisense treatment significantly lowered the levels of [^{3}H]citrulline by 32% ($P < 0.05$).

To further define the role of nNOS-2 in spinal morphine analgesia, we performed full morphine dose-response curves after administering antisense C spinally. The antisense treatment significantly shifted the dose-response curve over 2-fold, raising the ED_{50} from 4.3 mg/kg (2.9, 6.1) to 9.2 mg/kg (6.3, 13.3) in antisense-treated mice

Supraspinal treatments revealed similar results. Antisense C again blocked morphine analgesia (Fig. 4*b*), confirming a supraspinal role for nNOS-2 in morphine analgesia as well. However, all the probes targeting nNOS-1 given supraspinally were inactive against morphine analgesia, including those targeting both nNOS isoforms.

NOS and Morphine Tolerance. NOArg given systemically prevents morphine tolerance in a daily injection paradigm (11, 12). NOArg at a dose (1 μg, i.c.v.) that did not enhance morphine analgesia blocked the tolerance seen with repeated morphine administration ($P < 0.001$) (Fig. 5*a*). Antisense A at two different doses had a similar effect (Fig. 5*b*). Tolerance developed in the mismatch control group as rapidly as the control group, confirming the specificity of the response. An antisense oligodeoxynucleotide targeting inducible NOS was inactive.

We then examined a series of different antisense and mismatch oligodeoxynucleotides given supraspinally against morphine tolerance (Fig. 6*a*). Like the control group, tolerance to the fixed morphine dose developed in all the mismatch groups by day 5. In contrast, all the probes targeting nNOS-1 blocked the development of tolerance.

The marked reduction in morphine analgesia following intrathecal NOArg and antisense C administration makes it impractical to explore them in this paradigm. Although intrathecal antisense D administration, which selectively targets nNOS-1, had no effect on morphine analgesia, it prevented morphine tolerance (Fig. 6*b*). Again, the corresponding mismatch oligodeoxynucleotide was not active.

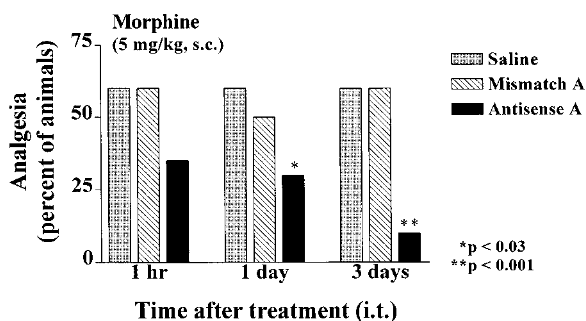

FIG. 3. Effects of spinal nNOS antisense A treatment on morphine analgesia. Groups of mice (n = 20–30) received saline, antisense A (5 μg, i.t.) or mismatch A (5 μg, i.t.) and were tested at the indicated time with systemic morphine (5 mg/kg, s.c.). Systemic morphine analgesia was significantly lowered only after 1 ($P < 0.03$) and 3 ($P < 0.001$) days.

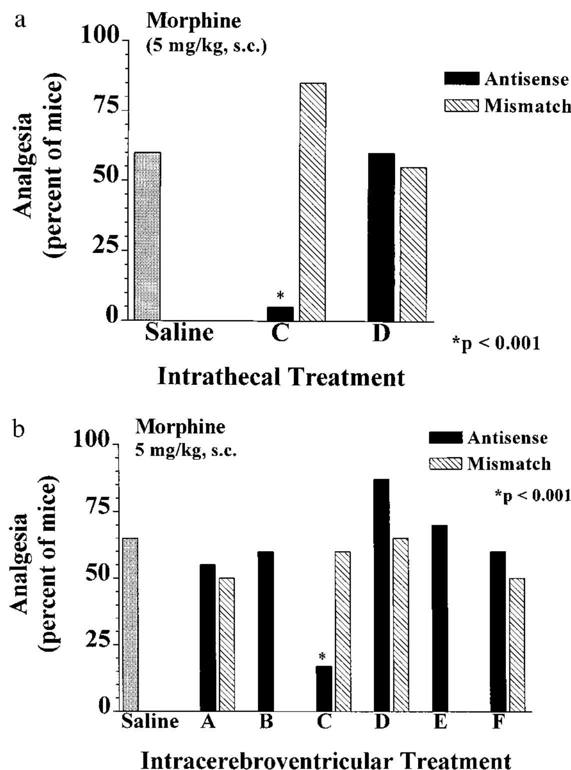

FIG. 4. Effects of nNOS antisense treatment on systemic morphine analgesia in naive mice. Three doses of saline or the indicated oligodeoxynucleotide (5 μg) were given on days 1, 3, and 5 either (*a*) i.t. or (*b*) i.c.v. Four hours after the last oligodeoxynucleotide treatment mice were tested with morphine (5 mg/kg, s.c.). Antisense C given either i.c.v. or i.t. lowered morphine analgesia significantly ($P < 0.001$) compared with saline controls. No significant changes were seen with any other antisense or any of the mismatch controls.

NOArg and Morphine Analgesia. Given systemically, NOArg does not affect morphine analgesia despite its ability to block and reverse morphine tolerance (11, 12). However, a different picture emerged when NOArg was given centrally. Supraspinal NOArg significantly enhanced systemic morphine analgesia while spinal NOArg markedly reduced systemic morphine analgesia (Fig. 7*a*) in a dose-dependent manner (Fig. 7*b*). NOArg also reduced spinal morphine analgesia and potentiated supraspinal analgesia, but only when both agents were given at the same site (Fig. 7*a*). The lack of NOArg activity when it was given into a different site than morphine confirmed that their interactions were localized to the region. These opposite NOArg actions indicated a predominance of the nNOS-1 system supraspinally and the nNOS-2 system spinally.

DISCUSSION

NO has a number of important actions in the central nervous system, including the modulation of morphine actions. Although our initial investigations found that NO plays a major role in the production of morphine tolerance, our current studies reveal a far greater complexity, with two nNOS isoforms responsible for opposing actions of NO on morphine analgesia.

As observed in other studies, antisense treatment downregulates nNOS mRNA levels by 50–75%, a level similar to

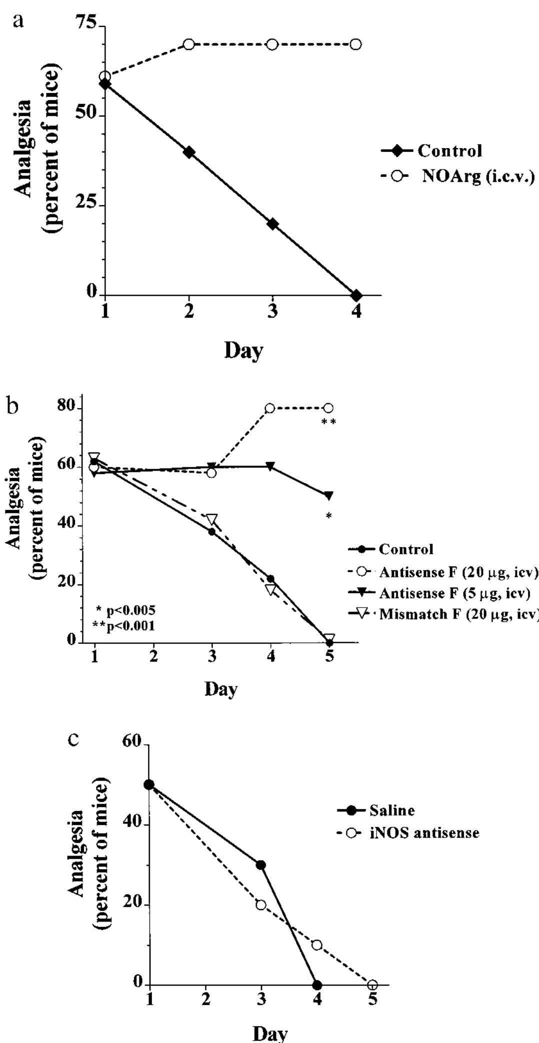

FIG. 5. Effect of NOArg and antisense on morphine tolerance. (*a*) Groups of mice received NOArg (1 µg, i.c.v.) or vehicle daily along with morphine (5 mg/kg, s.c.). Analgesia was tested at the indicated times. After 4 days of morphine, the NOArg group was significantly higher than the control ($P < 0.002$). (*b*) Groups of mice ($n \geq 10$) received the indicated saline, antisense F (20 or 5 µg, i.c.v.), or mismatch F treatment every other day for a total of four treatments, starting 3 days before the initiation of the chronic morphine dosing. On day 1, the day of the third antisense or mismatch treatment, all groups started receiving daily morphine injections (5 mg/kg, s.c.) and were tested for analgesia. On day 5, the mismatch and control groups were significantly different from both doses of antisense F (20 µg, $P < 0.001$; 5 µg, $P < 0.005$). (*c*) Groups of mice ($n = 10$) received three injections of saline or an antisense (5 µg, i.c.v.) targeting iNOS (5′-GAT CCT GCC GAT GCA GCG AG-3′; GenBank accession no. M92649) on alternate days. Mice started receiving morphine (5 mg/kg, s.c.) on the last day of antisense treatment. The time on the figure refers to the days of morphine treatment. The iNOS antisense had no effect upon the development of morphine tolerance.

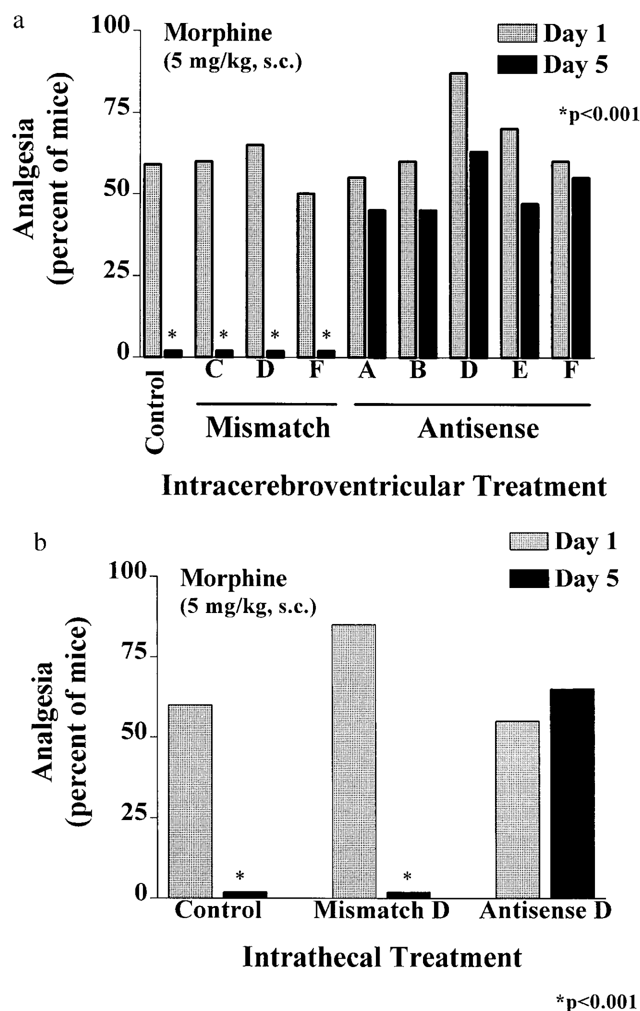

FIG. 6. Effects of supraspinal and spinal nNOS antisense on morphine tolerance. Groups of mice ($n \geq 20$) received three doses of the indicated oligodeoxynucleotide (5 µg) (*a*) i.c.v. or (*b*) i.t. every other day. Starting on the day of the last oligodeoxynucleotide treatment (day 1), the mice received daily morphine injections (5 mg/kg, s.c.). The analgesic activity is given on day 1 and day 5. The analgesic activity in the control and mismatch groups on day 5 are all significantly different ($P < 0.001$) from their own values on day 1. The responses of the antisense treated animals on day 5 do not differ from their values on day 1, but they do differ from the day 5 values of the control and mismatch groups ($P < 0.002$).

that seen with antisense studies on opioid receptors (46). This reduction in mRNA levels is accompanied by a smaller drop in enzymatic activity. Several factors may explain this difference. First, the decrease in mRNA levels would be expected to precede lower protein levels, partially explaining the lower drop in enzymatic activity. More important, the enzymatic assay does not have the selectivity of the antisense approach and a portion of the activity seen in these assays is likely to be due to isoforms other than nNOS.

The antisense oligodeoxynucleotide common to both isoforms lowers the mRNA levels of both to a similar extent. We anticipated that Antisense D would selectively lower nNOS-1 mRNA levels because the targeted sequence is unique to that splice variant. Targeting nNOS-2 was less certain because all the exons comprising nNOS-2 also are present in nNOS-1. To overcome this problem we targeted the only unique aspect of the nNOS-2 mRNA, the splice site. By limiting the number of bases on either side of the splice site, we are able to down-regulate nNOS-2 without appreciably affecting nNOS-1. This ability to selectively down-regulate individual splice variants *in vivo* provides a method to independently define their functional activities, as illustrated with our current studies. Equally important, this approach can readily be extended to many other proteins, particularly those within the central nervous system.

The antisense results exposed an unanticipated pharmacological complexity for NO/opioid interactions. nNOS-2 is important in modulating both spinal and supraspinal morphine analgesia. Selectively targeting nNOS-2 with antisense C lowers morphine's analgesic potency at both levels of the neuraxis. Although there is a dramatic loss of activity at single doses, this reflects only a 2- to 3-fold increase in ED_{50} values determined

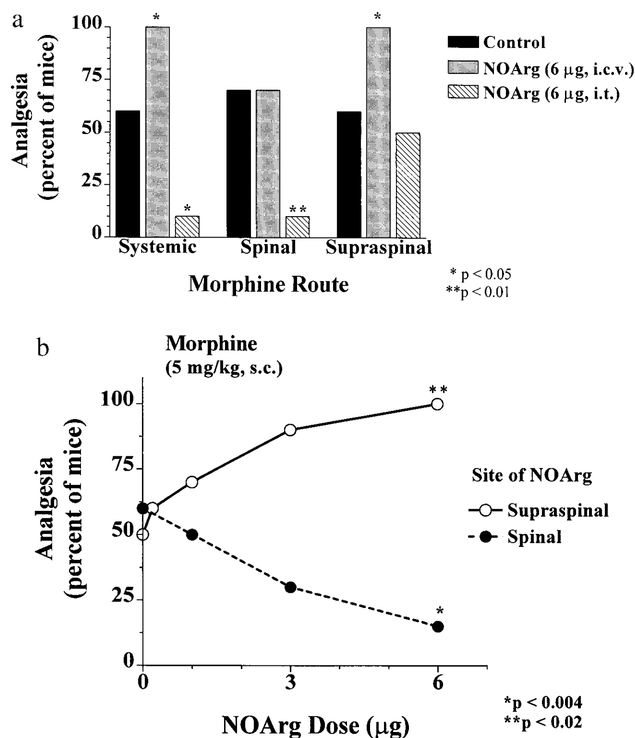

FIG. 7. Effects of central NOArg on morphine analgesia. (*a*) Groups of mice ($n \geq 10$) received NOArg (6 μg) either spinally or supraspinally. The effects of NOArg were then assessed following morphine given either systemically (5 mg/kg, s.c.), i.c.v. (250 ng) or i.t. (500 ng). Analgesia was assessed 30 min following systemic administration of morphine or 15 min after central administration. NOArg given alone in either location was inactive in the tailflick assay (data not shown). NOArg given i.c.v. significantly elevates systemic and i.c.v. morphine analgesia ($P < 0.05$). NOArg given i.t. significantly lowers systemic ($P < 0.05$) and i.t. morphine analgesia ($P < 0.01$). (*b*) Groups of mice received the indicated dose of NOArg either supraspinally (i.c.v.; $n = 10$) or spinally (i.t.; $n = 20$) and received morphine (5 mg/kg, s.c.) 30 min later. Analgesia was assessed 30 min following morphine. At 6 μg, i.c.v. NOArg significantly ($P < 0.02$) elevates and i.t. NOArg significantly lowers ($P < 0.004$) systemic morphine analgesia.

from dose–response studies. Thus, nNOS-2 appears to play a modulatory role rather than being an integral component of the analgesic circuit. Presumably, morphine would retain its analgesic activity with a complete loss of the nNOS-2 system, although its potency would be greatly lowered.

In contrast, morphine tolerance is modulated by nNOS-1 both supraspinally and spinally. Five different antisense probes targeting nNOS-1 given supraspinally all blocked morphine tolerance, as does the nNOS-1 selective antisense given intrathecally. Several of these probes down-regulate both nNOS-1 and nNOS-2 without affecting analgesia. Presumably the loss of the facilitating nNOS-2 system is compensated by the concomitant down-regulation of the inhibitory nNOS-1 system, yielding little net change in the response to morphine either acutely or with repeated administration, manifested as a loss of tolerance. Thus, these studies associate the two nNOS isoforms with opposing effects on morphine analgesia both spinally and supraspinally. nNOS-1 diminishes the analgesic actions of morphine while nNOS-2 enhances them.

The lack of effect of systemic NOArg on morphine analgesia (11, 12) probably reflects the simultaneous blockade of both facilitating and suppressive NO systems. NOArg has little selectivity among NOS isoforms and would be expected to block them all. If both facilitating and suppressive NO systems are simultaneously blocked, the net effect may be quite minimal. However, this lack of effect is no longer seen when NOArg is given either spinally or supraspinally. At the spinal level, NOArg inhibits morphine analgesia, suggesting the predominance of the morphine-facilitating nNOS-2 system. This corresponds well with the relatively high levels of nNOS-2 reported in the spinal cord (32). Supraspinal NOArg enhances morphine analgesia, implying that inhibitory nNOS-1 systems play the major role at this site.

In conclusion, the current study separates the functional roles of two alternatively spliced isoforms of nNOS. This approach is suitable for exploring other functional roles of nNOS in the central nervous system. The ability to design active probes spanning splice sites as well as probes targeting individual exons provides further validation of the antisense mapping approach and may prove useful for exploring the functional roles of a wide variety of proteins.

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