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Intracerebroventricular injection of antisense oligos to nNOS decreases rat ethanol intake

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Abstract

Nitric oxide (NO) has been implicated in alcohol drinking behavior using NO synthase (NOS) inhibitors that are nonselective of the different isoforms of NOS. In the brain, there are two constitutive isoforms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS). We used an antisense oligodeoxynucleotide directed against nNOS in ethanol dependent male Wistar rats to examine the specific contribution of nNOS in the control of ethanol intake. Rats were subjected to a free-choice situation water/ethanol (10% v/v) after chronic ethanol intoxication by inhalation of ethanol vapor. During the free-choice situation, rats were twice daily for 4 days intracerebroventricularly injected with either saline, or end-capped phosphorothioate-protected antisense or mismatch oligodeoxynucleotide (25 μg/4 μl per injection), or acamprosate (1 mg/kg body weight) as reference product for its anticraving properties. Our results showed that the antisense treatment, but not the mismatch treatment, reduced both ethanol intake and ethanol preference during treatment and posttreatment periods (by 25–30%) without alteration of the body weight gain. The antisense treatment, but not the mismatch treatment, also down-regulated nNOS mRNA levels (by 30%) and NOS activity in the hippocampus. The anticraving drug, acamprosate reduced both ethanol intake (by 58%) and ethanol preference. All these results suggest that nNOS is involved in the regulation of alcohol dependence.
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1. Introduction

Nitric oxide (NO) is a diffusible free radical that has been recognized as a biological messenger involved in several physiological and pathological functions. NO is produced from L-arginine by at least three isoforms of nitric oxide synthase (NOS) designated neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [15].

In the central nervous system, the predominant isoform is nNOS [7] and NO generated by this isoform acts as a neurotransmitter and a second messenger activating cGMP production [16]. NO has been implicated in various neurophysiological functions including feeding [32], drinking [8], anxiety [33] and synaptic plasticity [10]. An overactivation of nNOS is associated with *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity and neuronal death and it has been suggested that NO could be involved in ethanol-induced brain damage [11,26].

Several studies have emphasized a potential role for NO, not only in the neurotoxic effects of ethanol, but also in alcohol-seeking behavior. In this regard, NOS inhibition has been shown to impair rapid tolerance to ethanol [22], to potentiate the sedative–hypnotic effects of ethanol [1] and to alleviate some signs of ethanol withdrawal [2]. In addition, an implication of NO has been shown in alcohol-drinking behavior, since NOS inhibitors reduce ethanol preference and/or ethanol consumption in alcohol-dependent rats and in different strains of alcohol-preferring rats [9,36]. In some studies the effects of NOS inhibitors have been shown to be prevented by the coadministration of L-arginine, a NO precursor. In this regard, L-arginine prevented the effects of N^G-nitro-L-arginine (L-NNA) on rat ethanol intake [9] and the effects of N^G-nitro-L-arginine

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methyl ester (L-NAME) or 7-nitro indazole (7-NI) on the signs of ethanol withdrawal [40].

Most of the works that have showed an implication of NO in the control of ethanol consumption and in the development of different manifestations associated with ethanol consumption did use competitive inhibitors of NOS (N^G-substituted analogs of L-arginine). These chemical inhibitors are not specific for one isoform of the enzyme and do not permit to avoid in vivo side effects, i.e. vasoconstriction caused by inhibition of eNOS. 7-NI, described as an nNOS selective inhibitor, does not produce significant increase of blood pressure in vivo, but is reported to inhibit the three isoforms of NOS in vitro [4], and until recently no explanation for its in vivo selectivity was available [34].

An alternative approach to nonselective pharmacological inhibition of the enzyme NOS consists in the specific blockade of gene expression of a single isoform that can be achieved using antisense strategy in which antisense oligodeoxynucleotide binds specifically to the complementary region of the mRNA, preventing its translation by several mechanisms (RNase H degradation, inhibition of mRNA translation and impairment of nuclear transport) [17,24].

In the present study, we used this antisense approach, in vivo, to specifically assess the contribution of nNOS isoform in the regulation of ethanol dependence.

2. Methods

2.1. Materials

L-[2,3,4,5-^{3}H]arginine (69 Ci/mmol) was obtained from Amersham, Dowex AG50WX 8 resin (Na^+ form) was purchased from BioRad. Dithiothreitol, L-arginine, β-mercaptoethanol and Triton X-100 were obtained from Sigma. Fifteen-gauge stainless steel cannula and twenty-five-gauge needle were obtained from Leguellec (France). Wistar Ico:WI AF/Han rats were supplied by Iffa-Credo, 69210 France.

2.2. Ethanol treatment

Adult male Wistar rats weighting 220–240 g at the beginning of the experiment were chronically intoxicated by inhalation of ethanol vapor during 4 weeks as previously described [3]. Ethanol concentration was increased from 15 to 23 mg/l of air in successive steps of 1 mg/l every 2–4 days so that the average blood alcohol levels (BALs) was continuously increasing. BALs were regularly determined to permit the adjustment of ethanol vapor concentrations within the inhalation chambers. Blood samples (0.1 ml) were taken from the retro-orbital sinus of anesthetized animals (with diethyl ether) and tested by an alcohol dehydrogenase enzymatic method (Behring kit). Rats had free access to food and water during the alcohol inhalation treatment. At the end of 4 weeks of ethanol inhalation the intoxicated animals were given a two-bottle choice (water/10% ethanol). Daily consumption (expressed as a percentage of total fluid intake for ethanol preference and as ethanol intake g/kg body weight) was calculated for each rat. After 7 days, rats were implanted with stainless-steel guide cannula into the left lateral ventricle and were allowed to recover for 7 days. Rats were then randomly split in function of their ethanol consumption to obtain four homogeneous groups (five rats per group) consuming approximately 7 g/kg body weight/day of ethanol. These four groups were then treated with either saline (control) or antisense or mismatch or acamprosate.

Three groups (five rats per group) that have been exposed to chronic ethanol and three other groups that have never been exposed to ethanol were treated with either saline (control) or antisense or mismatch and were used for biochemical and/or molecular studies.

The procedures described comply with ethical principles and guidelines for care and use of laboratory animals adopted by the European Community, law 86/609/EEC.

2.3. Design of antisense oligodeoxynucleotides

Based on the cDNA sequence for the rat nNOS (Genbank accession number X59949; [7]), two 19-mer end-capped phosphorothioate modified oligodeoxynucleotides were designed. The antisense was targeted to the area of NOS 1 cDNA sequence that bridges the initiation codon (from −11 to +8; 5′-T * TCTCTTCCATGGTATCT * G-3′; * = phosphorothioate linkage). Mismatch sequence oligodeoxynucleotide (5′-T * TCACTTCGATGCTATAT * T-3′) and saline served as controls.

The oligodeoxynucleotides were synthesized and purified by reverse phase high-performance liquid chromatography methods by Genosys (UK). Oligodeoxynucleotides were dissolved in saline (NaCl 0.9%) and stored at −20°C until used.

2.4. Intracerebroventricular injections

Rats were anaesthetized with chloral hydrate (400 mg/kg body weight ip) and were stereotaxically implanted with a 15-gauge stainless steel cannula into the left lateral ventricle (0.8 mm posterior to the bregma, 1.5 mm lateral to midline, 3.6 mm ventral from dura). The cannula was secured to the skull with three stainless steel screws and dental cement.

Injections (4 μl) were made with a 10-μl Hamilton syringe connected to a 25-gauge needle by a catheter. The cannula implantation was made after 7 days of free-choice situation, and the animals were allowed to recover for 7 days prior to the start of different treatments. Rats were intracerebroventricularly injected twice daily for 4 days with either oligodeoxynucleotides (25 μg per injection) or acamprosate (1 mg/kg body weight) or saline (control group).

2.5. NOS activity determination

NOS activity in hippocampus was determined by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline as previously described [32]. Briefly, the appropriate tissue was homogenized in 2 ml of the HEPES buffer (20 mM, pH 7.4) containing 0.5 mM EDTA and 2 mM β-mercaptoethanol using an ultrasonic dismembrator for 10 s at setting 8 at 4°C. The homogenate was centrifuged at 18 000 g for 30 min at 4°C. The supernatant was applied to a 0.5 ml of Dowex AG50WX-8 (Na^+ form) column, preequilibrated with 1 ml of the homogenizing buffer to remove the endogenous L-arginine. The column was eluted with 0.5 ml of the homogenizing buffer. An aliquot of the elute containing the enzyme (150–250 μg protein) was added to the incubation medium containing 50 mM HEPES buffer, pH 7.4, 0.5 mM β-mercaptoethanol, 1 mM dithiothreitol, 2 mM NADPH, 0.5 mM $CaCl_2$ and 2.5 μM L-arginine containing 0.1 μCi/ml of [^{3}H]arginine. The incubation was carried out in triplicate for 30 min at 24°C. The blanks were run similarly but without NADPH and $CaCl_2$. The reaction was stopped by addition of 0.5 ml of stop buffer (HEPES 20 mM, pH 5.5, containing 2 mM EDTA). The contents of the incubation tubes were transferred to 0.5 ml of Dowex AG50WX-8 (Na^+ form) resin. [^{3}H]citrulline formed was eluted from the column using 2 ml of distilled water. Eluate (0.5 ml) was added to a scintillation vial containing 5 ml of Optiphase HiSafe Wallac scintillation fluid (EGG). The radioactivity in the samples was determined in a LKB Wallac RackBeta 1211 liquid Scintillation Counter. The efficiency of the counter was calculated for each experiment (approximately 30%). The protein concentration in the samples was measured by the method of Lowry et al. [29]. NOS activity was expressed as picomoles of [^{3}H]citrulline formed per minute per milligram protein.

2.6. Quantitative analysis of nNOS mRNA levels

The method was based on competitive polymerase chain reaction (PCR) [37], in which RNAs were reverse-transcribed and amplified in the presence of an internal standard consisting of the same target mRNA, synthetized with a deletion of about 100 bases to allow electrophoretic separation as previously described. An internal standard RNA was synthesized by transcription of the cDNA coding for the rat nNOS corresponding to the region +1 to +500 [7]. A deletion of 110 bases was introduced by double cleavage with the restriction endonuclease Hinc II, at the +135 and +245 sites. Transcription with T7 RNA polymerase from recombinant linearized pGEM-3Zf(+) plasmid was achieved using the Ampliscribe kit (Epicentre Technologies, Madison, WI, USA).

Total RNAs were extracted using the RNA Insta-Pure System (Eurogentec, Belgium) according to the manufacturer's procedure. The concentration of total RNAs were determined by absorption at 260 nm and their integrity confirmed by electrophoresis through 1.5% agarose gels.

Target RNAs (0.2 μg total RNA per sample) were converted to cDNAs using 5 units of reverse transcriptase from Moloney-Murine leukemia virus (Stratascript, Stratagene, Germany) for 2 h at 42°C in 5 μl of 50 mM Tris–HCl, pH 8.3, containing 10 units RNAse inhibitor (Promega), each deoxynucleoside triphosphate (d-ATP, d-CTP, d-GTP and d-TTP) 1 mM, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 800 nM reverse primers plus various amounts (0.01–1 pg) of synthetic deleted RNAs. Sequence of the reverse primer was 5′-CAGGTCTGAGATGATCACGG (nucleotides 486–467). Amplification was then performed in the same tubes in 25 μl of 10 mM Tris–HCl, pH 8.3, containing 1 unit of Taq polymerase (Hi-Taq, Bioprobe System, France), 2 mM $MgCl_2$, 35 mM KCl, 0.1% Triton X-100, 0.2 mM each deoxynucleoside triphosphate, and 200 nM of the primer 5′-ACGTCTGACAAGCTGGTGAC (nucleotides 1–20). The amplification profile involved four linked files as follows: 5 min at 94°C for 1 cycle; 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 28 cycles and finally 8 min at 72°C for 1 cycle. Preliminary experiments with different concentrations of $MgCl_2$ and variable numbers of PCR cycles were performed in order to obtain the best yields and linearity of the relationship for the calculation of specific mRNA. PCR products (376 base pairs in size for the synthetic deleted RNA and 486 base pairs for NOS 1-mRNA) were electrophoresed in 1.5% agarose gel stained with ethidium bromide and quantified with the gel analyzer The Imager (Appligene Oncor, France) and NIH Image 1.44 analysis Software. RNA levels are expressed as attomoles of synthetic RNA standard per microgram of tissue total RNA. Correction was made to account for the respective sizes of native mRNA and synthetic deleted RNA.

2.7. Statistics

Results are expressed as means ± S.E.M. Statistically, these data were tested within a group of experiments (i.e. control, antisense, mismatch and acamprosate) by means of one-way ANOVA. Post hoc analysis of significant effects were performed using Neuman–Keuls tests for multiple comparisons, to compare the effects of each treatment at different periods (during the treatment and the 3 weeks after the treatment) versus the respective pretreatment period.

3. Results

The BALs were 233 ± 13 mg/dl at the end of the 4 weeks of ethanol intoxication by ethanol vapors. Following this ethanol intoxication period, in the present experiment, when given free access to ethanol and water, rats consumed an average of 7.24 ± 0.932 g/kg body weight per day and the ethanol preference was 82 ± 6.9%.

Daily ethanol consumption in the control group (NaCl 0.9%) expressed as absolute ethanol intake (g/kg body weight) is significantly increased [$F(4,99)=4.33$, $P<.005$] only during the treatment period ($P<.05$, Neuman–Keuls Post hoc analysis), but the ethanol preference is not modified all along the experiment [$F(4,99)=0.95$, $P>.05$] (Fig. 1A). However, the treatment did not modify the ethanol consumption during the treatment period in the other groups (antisense, mismatch or acamprosate).

The antisense treatment significantly decreased ethanol intake [$F(4,99)=5.49$, $P<.001$] during the 2 weeks after the treatment period compared to the pretreatment period ($P<.05$, Neuman–Keuls post hoc analysis) (Fig. 1A). Moreover, the antisense treatment also significantly decreased ethanol preference [$F(4,99)=3.56$, $P<.01$] during the treatment period ($P<.05$, Neuman–Keuls Post hoc analysis), the first week ($P<.01$, Neuman–Keuls Post hoc analysis) and the second week ($P<.05$, Neuman–Keuls Post hoc analysis, Fig. 1B). The mismatch treatment significantly decreased ethanol intake [$F(4,99)=4.93$, $P<.001$] but only in the second and the third week after the treatment period ($P<.05$, Neuman–Keuls Post hoc analysis, Fig. 1A) and had no effect on ethanol preference $F(4,99)=2.39$, $P>.05$ (Fig. 1B).

The acamprosate treatment significantly decreased ethanol intake [$F(4,99)=4.56$, $P<.01$] during the first week of

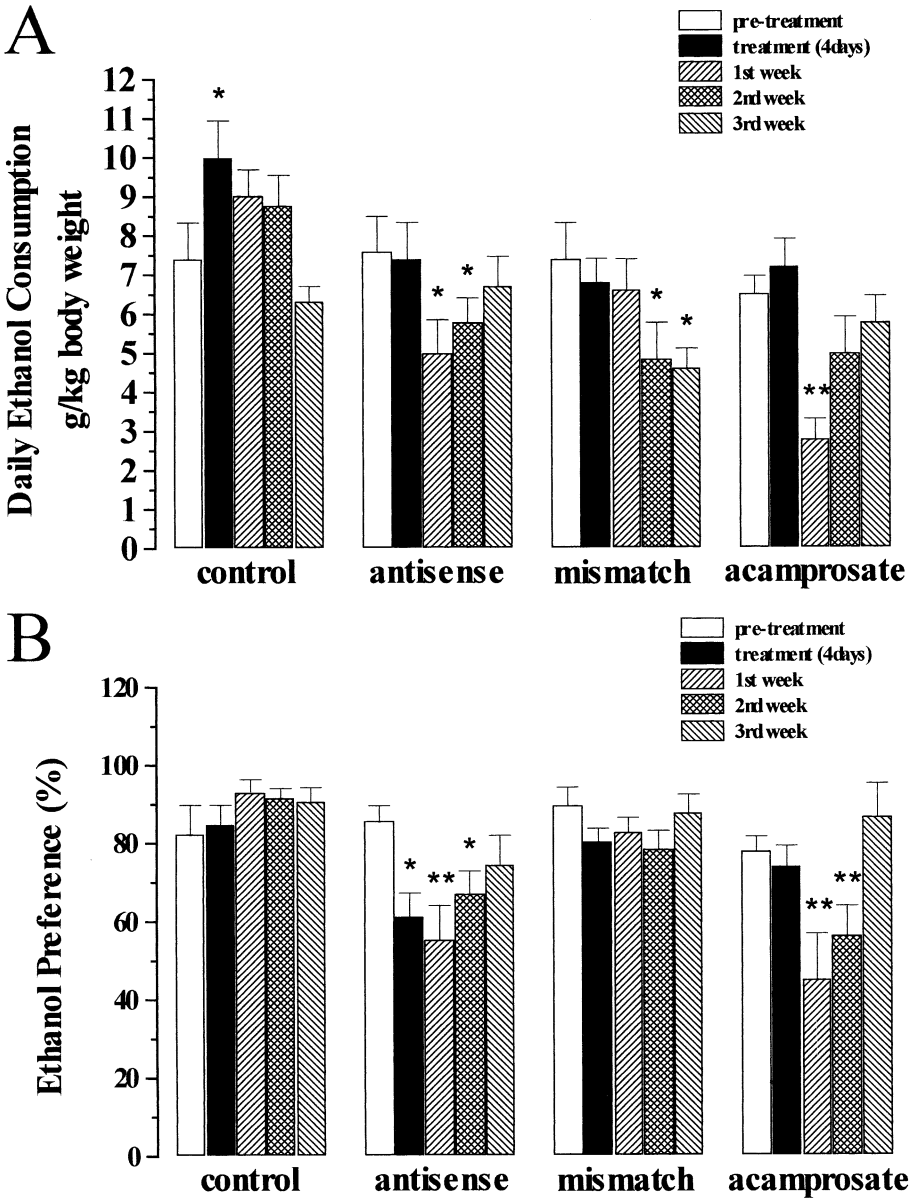

Fig. 1. (A) Daily ethanol consumption expressed as absolute ethanol intakes (g/kg body weight). (B) Ethanol preference was calculated as the total ethanol intake (ml)/total fluid (alcohol + water) intake (ml) × 100. Rats were twice daily for 4 days intracerebroventricularly injected with either saline (control) or oligodeoxynucleotides (antisense or mismatch) (25 μg/4 μl per injection) or acamprosate (1 mg/kg body weight). The data are the means ± S.E.M. of five rats per group. * $P<.05$, * * $P<.01$ compared to respective pretreatment period (Neuman–Keuls post hoc test following a one-way ANOVA).

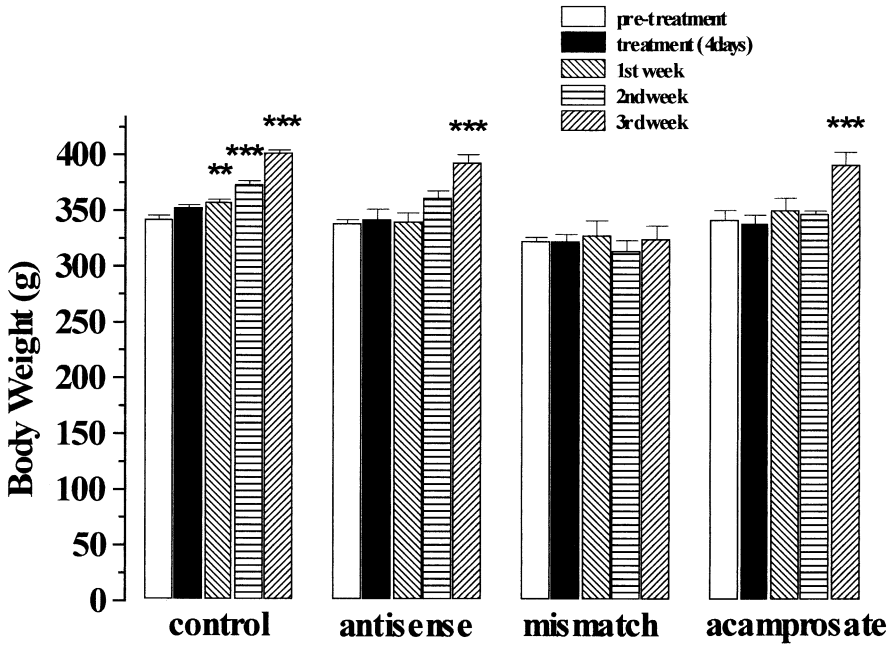

Fig. 2. Body weight (g). The data are the means ± S.E.M. of five rats per group. $**P<.01$, $***P<.001$ compared to respective pretreatment period (Neuman–Keuls post hoc test following a one-way ANOVA).

posttreatment ($P<.01$, Neuman–Keuls Post hoc analysis, Fig. 1A) and ethanol preference during the 2 weeks after the treatment period compared to the pretreatment values ($P<.01$, Neuman–Keuls Post hoc analysis, Fig. 1B).

As illustrated in Fig. 2, body weights were significantly increased during the experiment in control, antisense and acamprosate groups [$F(4,109)=74.20$, $P<.0001$, $F(4,109)=10.13$, $P<.0001$, $F(4,109)=4.10$, $P<.005$, respectively], whereas the mismatch group did not gain body weight [$F(4,109)=0.39$, $P>.05$].

The antisense treatment significantly decreased both NOS activity [$F(2,14)=6.10$, $P<.05$; $P<.05$ compared to mismatch values] and nNOS mRNA levels [$F(2,14)=9.29$, $P<.05$; $P<.05$ compared to control values] in the hippocampus of rats that had not been previously exposed to ethanol, whereas mismatch had no significant effect (Fig. 3). In ethanol-treated rats, the antisense treatment decreased NOS activity [$F(2,14)=2.86$, $P<.05$; $P<.05$, Neuman–Keuls Post hoc analysis compared to ethanol values] whereas mismatch had no significant effect. In these etha-

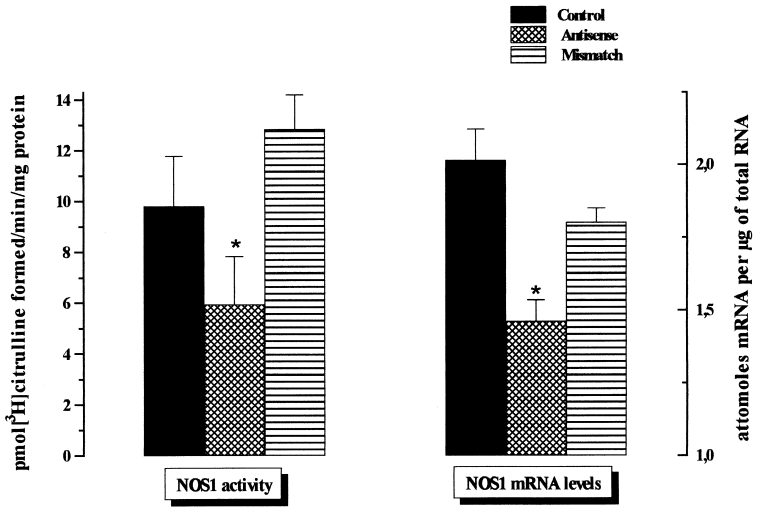

Fig. 3. NOS activity (left panel) and nNOS mRNA levels (right panel) measured in rats after 4 days of treatment with either saline (control) or oligodeoxynucleotides (antisense or mismatch, 25 μg/4 μl per injection, twice daily for 2 days). These rats have never been exposed to ethanol. Activity and mRNA levels were measured in rat hippocampus 2 h after the last injection. $*P<.05$ compared to mismatch treatment (left panel) and $*P<.05$ compared with control (right panel) (Neuman–Keuls post hoc test following a one-way ANOVA).

nol-treated rats the NOS activity was 9.40 ± 0.62, 5.97 ± 1.29 and 9.10 ± 1.18 pmol of [^{3}H]citrulline formed per minute per milligram protein in ethanol, ethanol plus antisense and ethanol plus mismatch groups, respectively.

4. Discussion

In the present study, we used end-capped phosphorothioate-protected antisense that has been shown to be efficient in vivo and to present a low toxicity in comparison with the fully modified phosphorothioate oligodeoxynucleotides [19,35]. Moreover, since the predominant mode of degradation of oligodeoxynucleotides is the attack by α-exonucleases, we chose to use end-capped phosphorothioate-protected antisense that has been shown to be resistant to this form of attack both ex vivo and after intracerebroventricular administration [41].

The anticraving drug acamprosate [28] decreased daily ethanol consumption by 58% during the first week after the treatment period and ethanol preference. To our knowledge, this is the first time that the effect of acamprosate on ethanol drinking behavior is investigated through an intracerebroventricular administration. Its effect is more rapid and pronounced than that observed using an intraperitoneal injection, suggesting the importance of the bio-availability of the drug for its therapeutic action [6]. The efficacy of acamprosate to decrease ethanol intake in this experiment confirm the rat's ethanol dependence and their sensitivity to anticraving drugs.

Our results showed that the antisense treatment decreased both absolute ethanol intake and ethanol preference and rats normally gained body weight all along the experiment as observed in the control group. The antisense treatment decreased ethanol consumption both during and after treatment. This effect is associated to a decrease of hippocampal NOS activity and nNOS mRNA levels as shown on Fig. 3 (and described in the Results section), indicating that the behavioral effect observed can be associated to the selective inhibition of the nNOS. The hippocampal NOS activity is decreased in rats that have been previously exposed to ethanol or not, showing that this decrease is due to the antisense treatment. We previously showed that chronic ethanol exposure has no significant effect on hippocampal NOS activity [32]. The NOS activity and nNOS mRNAs levels were measured in the hippocampus, a brain area that is implicated in the regulation of ethanol-drinking behavior [14]. The mRNA levels quantification was realized 2 h after the last antisense injection and we observed only a 25% decrease in mRNA levels. Several hypotheses can be argued: the turn-over of nNOS mRNA is rapid or the blockade of the translation could also induce an increase of the transcription as a regulatory adaptation or finally, more time is needed for an antisense accumulation and a better efficacy. This latter hypothesis could explain the fact that the effect of the antisense treatment is observed during 2 weeks following the treatment period. Moreover, it is possible that antisense functions essentially via a hybrid-arrested translation process that could explain why mRNA levels are slightly decreased. In this context it is interesting to note that other experiments using the in vivo antisense approach observed either an unchanged [38] or an elevated [27] mRNA content despite pronounced behavioral effects.

On the other hand, the decrease of nNOS mRNA levels by antisense treatment is not accompanied by a higher drop in hippocampal NOS enzymatic activity, indicating that the existing pool of the constitutive nNOS protein remained active. The antisense treatment inhibits the production de novo of the targeted protein. Because antisense oligonucleotides target RNA and not protein, biological consequences that result from the inhibition of a particular gene product is dependent on the half-life of the encoded protein product.

Another study has reported that phosphodiester antisense treatment in mice (single icv injection 20 μg/2 μl) down-regulates nNOS mRNA levels by 50–75% and NOS activity by only 32%, and has suggested that this difference could be explained by the lack of selectivity of enzymatic assay for the different constitutive NOS isoforms [23]. However, in this study the results of the NOS activity are presented as nNOS activity assuming that nNOS is the predominant isoform in the brain. The protein turn-over of the nNOS has not been determined, but with our procedure our results suggest that 4 days of antisense treatment is sufficient to achieve a partial reduction of hippocampal NOS activity at the fourth day of treatment. In this regard, the reduction of ethanol intake and ethanol preference observed during the first week and/or the second week of posttreatment could be caused by an accumulation of the antisense oligodeoxynucleotide and its subsequent more long-term effect on nNOS protein.

The effect of nNOS antisense oligodeoxynucleotide on ethanol consumption is specific of ethanol intake since the ethanol preference is also decreased. Conversely, the mismatch treatment decreased absolute ethanol intake but had no effect on both ethanol preference, NOS activity and nNOS mRNAs levels. This effect on ethanol intake that is only observed during the second and the third week after the treatment period can be attributed to side and/or toxic effects because rats did not normally gain body weight at the end of the experiment. Several studies have emphasized a potential toxicity of oligodeoxynucleotides [41,42]. An interaction of the nNOS blockade with alcohol metabolism can be ruled out [8,36].

The blockade of nNOS activity using nonselective NOS competitive inhibitors L-NAME and L-NNA or selective nNOS competitive inhibitor 7-NI [4] has also been described to decrease ethanol intake during treatment [5,9,36]. These studies have not reported an effect after the treatment period. A transient inhibition of NOS activity has been shown after intraperitoneal injection of 7-NI, suggesting that a posttreatment behavioral effect with this pharmacological approach can be ruled out. Furthermore, the NOS inhibition by 7-NI

(30 mg/kg ip) (maximal effect 0.5 h postinjection) has been reported to be transient with complete recovery at 24 h. Moreover, repeated intraperitoneal injections of 7-NI (30 mg/kg, every 4 h for 20 h) inhibited NOS activity at 24 h by 51–61% [30]. We showed in the present study that the antisense technology may prolong NOS inhibition and could be a good tool to study the effects of the specific inhibition of nNOS on alcohol-seeking behavior.

Only a few studies have examined the effects of ethanol on nNOS protein levels and activity. The protein level and enzymatic activity of nNOS have been shown to be differentially regulated depending on the rat brain regions after chronic ethanol exposure. For example, in hippocampus it has been reported that the protein level is decreased [13] but the kinetic parameters (K_M and V_{max}) are not significantly altered [32]. The effects of ethanol in vitro on the nNOS activity is not clearly demonstrated, since it has been reported that ethanol either decreased the rat cerebellar NOS activity in a dose-dependent manner [12] or had no effect in mice cerebellar (and in various other brain regions) homogenates [20].

A number of previous studies suggest that some of the rewarding effects of ethanol may be mediated by nNOS. Thus, the ethanol-induced conditioned place preference is completely blocked by 7-NI in mice [21] and the lever pressing for ethanol is reduced by 7-NI in a dose-dependent manner in rats [39]. However, it has been shown that neither L-NAME nor 7-NI produced discriminative stimulus effects similar to ethanol in rats [18,25].

Several evidences suggest that ethanol affects nitric oxide system and nitric oxide agents affect ethanol intoxication and withdrawal, but much is unknown. A major type of evidence comes from interactions between ethanol and glutamate systems that are known to stimulate nitric oxide synthesis. Our results indicate that the study of the NMDA downstream pathway using an antisense strategy is promising, despite some toxic effects that can be observed.

In conclusion, the present findings demonstrate for the first time that the specific inhibition of nNOS by an antisense technique caused a significant reduction in ethanol intake and ethanol preference. The involvement of the neuronal isoform of NOS in the regulation of ethanol dependence could explain behavioral and degenerative disorders associated to chronic ethanol consumption. The study of new specific pharmacological tools of nNOS-NMDA receptor pathway could be promising therapeutic or preventive agents for alcohol craving.

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