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Formalin-induced changes of NMDA receptor subunit expression in the spinal cord of the rat

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Summary. Using RT-PCR, the present study investigated the effects of formalin administration on mRNA expression coding for NMDA receptor (NR) subunits and splice variants in rat lumbar spinal cord. Subsequent to formalin injection (5%; subcutaneously) into the hind paw of Sprague-Dawley rats, the animals exhibited the typical biphasic behavioural pain response. Spinal cord (L3-6) was prepared six hours after formalin injection. In controls, NR1-b predominated over NR1-a, and NR1-2 and NR1-4 exceeded over NR1-1 and NR1-3, respectively. Regarding the NR2 subunit expression in controls, NR2B exhibited the highest expression, followed by decreasing proportions of NR2C, NR2A, and NR2D. Formalin treatment did not affect NR1 splice variant expression but significantly increased and decreased the proportion of NR2A and NR2C, respectively. In summary, the present data demonstrate adaptive changes in the NR subunit expression pattern in rat spinal cord due to formalin injection.

Keywords: Formalin – NMDA receptors – NR1 splice variants – NR2 subunits – Rat spinal cord – Pain

Introduction

NMDA receptors (NR), a pharmacological subclass of glutamate receptors, are fast-acting, ligand and voltage-gated (by extracellular Mg^{2+}) ion channels which are highly permeable for calcium (Vallano et al., 1998; Parsons et al., 1998). Presumably, natively expressed NR form heteromeric tetramers containing two NR1 subunits in combination with subunits of the NR2 family (NR2A-D) (Behe et al. 1995). Alternative splicing generates eight splice variants of the NR1 subunit (Laurie and Seeburg, 1994; Zukin and Bennett, 1995). The variants arise from splicing at three exons: exon 5 encoding a 21 amino acid sequence in the extracellular N-terminal domain (designated as the N1 cassette), and adjacent exons 21 and 22 encoding a 37 (C1 cassette) and 38 amino acid sequence (C2 cassette), respectively, in the intracellular C-terminal domain. The pharmacological properties of NR are largely influenced by their subunit composition. In particular, the NR2 subunits confer functional variability to the receptor (Monyer et al., 1994; Vallano et al., 1998; Monaghan et al., 1998).

Spinal cord NRs (Tölle et al., 1993, 1995; Luque et al., 1994) have gained attention in particular for their involment in nociception (Dickenson, 1997). NR antagonists have been shown to block the increase in excitability of spinal cord neurones induced by stimulation of nociceptive afferent fibres, particularly C-fibres (Dickenson and Sullivan, 1990; Woolf and Thompson, 1991).

The formalin test is a commonly used animal model of nociceptive activation (Coderre et al., 1990). A subcutaneous injection of formalin into the hindpaw of the rat causes an immediate and intense increase in the spontaneous activity of C fibre afferents and evokes a typical behaviour indicative of pain, such as lifting, flinching, licking or biting of the injected paw. The behavioural response to formalin is biphasic, with an early short phase followed by an extended tonic phase (Dubuisson and Dennis, 1977; Wheeler-Aceto et al., 1990). Systemic application of NR antagonists affects predominantly the late phase of formalin pain (Elliott et al., 1995; Chaplan et al., 1997). Using reverse transcriptase polymerase chain reaction, the present study investigated NR subunit expression in lumbar spinal cord of control and formalin-injected rats.

Materials and methods

Formalin test

Male albino rats (Sprague Dawley; 135–170 g of body weight) delivered by a commercial breeder (Charles River) were used. The animals were housed under standardized conditions and were given tap water and a diet of standard laboratory ad libitum. Both were withdrawn during the test. There were at least five days between delivery of the animals and the test day. Nociceptive behaviour was induced by subcutaneous injection of 50 μl of 5% formalin into the right hind paw according to the method described by Dubuisson and Dennis (1977). The behaviour of individually placed animals was observed for a period of 30 min starting directly after formalin injection. Characteristic pain behaviour, i.e. lifting, flinching, licking/biting of the injected paw were recorded and scored according to Coderre et al. (1993). Animals injected with isotonic saline were used as controls.

Molecular biology

Total RNA was prepared from lumbar spinal cord (L3-6) of adult control and formalin-treated rats using the TRIZOL reagent (Life Technologies) according to the manufacturers protocol. After photometrical quantification, the quality of the RNA was tested by denaturing formaldehyde gel electrophoresis. Prior to cDNA-synthesis the RNA was treated with DNase I under the following conditions: 1 μg of RNA was incubated for 15 min at 37°C in a total volume of 10 μl containing 1× first strand buffer (Life Technologies) and 1.5 units DNase I (Life Technologies). The DNase I was inactivated by addition of 1 μl of 25 mM EDTA solution and subsequent incubation for 10 min at 65°C. First-strand cDNA was synthesised by reverse transcription of 1 μg total RNA with 100 u Superscript™-II reverse transcriptase (Life Technologies) in first strand buffer containing 12.5 mM dithiothreitol, 500 μM of each dNTP, 5 μM pd$(N)_6$ (Pharmacia Biotech), and 20 u RNase inhibitor (Stratagene) according to standard protocols. The reaction was stopped by heating at 70°C for 15 min. PCR amplification of NR1 splice variants was performed in a final volume of 25 μl PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 0.5 μl cDNA, 1 u Ampli-Taq DNA Polymerase (Perkin Elmer), 200 nM of each primer, 200 μM of each dNTP, and 1 mM $MgCl_2$ on a PTC-200 Thermocycler (MJ Research). For the C-terminal splice variants of NR1, a common sense primer (5′-AAG CTG CAC GCC TTT ATC TG-3′) and various antisense primers were used (exon 22-containing isoforms, NR1-1 and NR1-2: 5′-CCC TCC TCC CTC TCA ATA GC-3′; exon 22-deleted isoforms, NR1-3 and NR1-4: 5′-CTG ACC GAG GGA TCT GAG AG-3′) (Allgaier et al., 1999). The amplification conditions were: 94°C for 5 min followed by 35 cycles of denaturation (94°C for 30 s), annealing (58°C for 30 s), and extension (72°C for 1 min), and a final extension step at 72°C for 10 min. Predicted product lengths were: NR1-1, 608 bp; NR1-2, 498 bp; NR1-3, 583 bp; NR1-4, 473 bp. The N-terminal splice variants NR1-a and NR1-b were detected using the sense primer 5′-TCA GCG ACG ACC ACG AGG GAC G-3′ and the antisense primer 5′-TTG TAG ATG CCC ACT TGC ACC A-3′ yielding in 667 bp (NR1-b) and 604 bp (NR1-a) amplification products (Allgaier et al., 1999), respectively. PCRs were run with initial denaturation at 94°C for 3 min, 35 cycles of denaturation (94°C for 30 s), annealing (64°C for 30 s), and extension (72°C for 1 min), and a final extension at 72°C for 10 min. Amplified products were separated by agarose gel electrophoresis (2%) and visualised under UV light after staining with ethidium bromide. Amplification products of the C-terminal splice variants were vacuum-blotted to a positively charged nylon membrane (PALL) and hybridised with a digoxygenin-labelled oligonucleotide 5′-GCC ACC AGC ATG AAG ACC CCT GCC ATG TTC TCA AAA GTG A-3′ as described previously (Allgaier et al., 1999). Hybridization was performed in 5 × SSC, 0.02% SDS, 0.1% lauroylsarcosine, and 1% blocking reagent (Roche Diagnostics) at 60°C. After stringent washing (0.1× SSC at 60°C) the specific binding of the probe was detected by chemiluminescence with CSPD and analyzed using a Diana II Digital Image Analyzer (Raytest). Fluorescent/chemiluminescent signals allowed an estimation of the expression ratios of the respective NR1 subunits.

Co-amplification of the cDNA fragments of the NR2A-D subunits was carried out by a semi-nested hot-start PCR in two reaction steps. Primers sequences and conditions of the first round were: NR2 sense, 5′GGGTGATGATGTT(TC)GT(GC)ATG-3′; NR2 antisense, T(GC)CTG(TC)TGGATCATGAAGGC-3′; initial denaturation (94°C for 3 min) followed by 25 cycles (94°C for 30 s, 53°C for 30 s, 72°C for 40 s) and a final extension (72°C for 10 min) (Flint et al., 1997). After dilution, the PCR product was reamplified using the 5′primer of the first round and a nested 3′primer (5′ATGAC(AC)GC(AG)AAGAAGGCCCA-3′) yielding a 247 bp amplification product. Both PCR reactions were performed in the presence of 400 nM of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 1 u Amplitaq DNA Polymerase (Perkin Elmer) in a final volume of 25 μl PCR buffer. The 247 bp PCR products were quantitatively analysed by slot-blot hybridization with digoxigenin-labelled oligonucleotides specific for NR subunits 2A, 2B, 2C, and 2D. A serial dilution (1:2; 1:4; 1:8) of each PCR product (sample) along with a serial dilution of the respective cloned NR2 subunit standards (100–3.125 ng) was transferred by a Bio-dot SF apparatus (BioRad) onto one of four different nylon membranes (Flint et al., 1997). The blots were hybridized at 55°C overnight, washed (NR2A,C,D: 0.2 × SSC; NR2B: 0.1 × SSC; 55°C) and detected immunologically (see above). The relative percentages of subunits NR2A-D were calculated for each sample by comparing the intensity of signals for PCR products and plasmid standards.

Statistics. All data are given as arithmetic means ± S.E.M. The significance of differences between the examined groups was determined by Student's t-test.

Results

Formalin-induced pain responses

Subsequent to formalin injection, the animals exhibited the typical pain behaviour including lifting, flinching, licking and biting of the injected paw. The formalin responses were biphasic, with an early short phase of intense pain within 10 min, followed by an extended tonic phase of moderate pain lasting from 15–60 min after injection (data not shown).

Expression of NR1 mRNA splice variants

Six hours after formalin injection, the animals were sacrificed and spinal cord (L3-6) homogenates were prepared as described in Materials and methods. Specific primer pairs were used to detect the various N-terminal (NR1-a, NR1-b) and C-terminal (NR1-1, NR1-2; NR1-3, NR1-4) splice variants of the NR1 subunit by RT-PCR. In control animals, NR1-b (con-

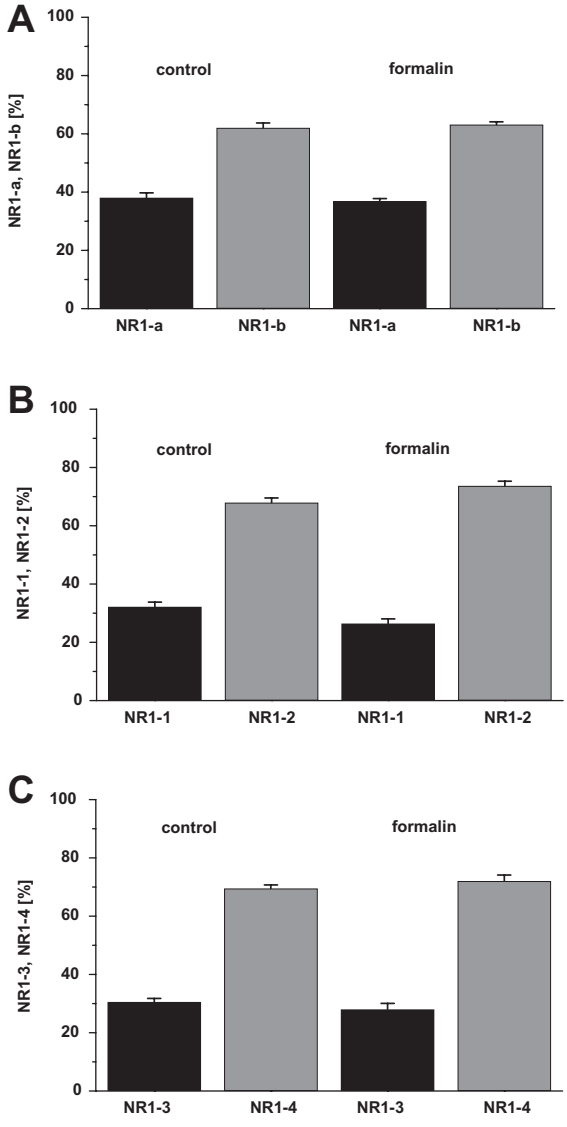

Fig. 1 A–C. NR1 splice variant expression in the spinal cord of control and formalin-treated rats. Six h after formalin treatment (5%), the spinal cord was prepared and RT-PCR was performed subsequently; cDNA products were separated by agarose gel electrophoresis (2%). (**A**) The ratio of the 3′-splice forms estimated under UV light after ethidium bromide staining. (**B**,**C**) The ratio of the 5′-splice forms determined by chemiluminescence analysis of the PCR products subsequent to Southern blot hybridisation. Means ± SEM of 8 experiments from 4 animals are given for each group

taining E5) showed a 1.6 fold higher expression than NR1-a (lacking E5) (Fig. 1A). Considering the C-terminal isoforms, NR1-2 (lacking E21 but containing E22) and NR1-4 (lacking E21 and E22) exceeded NR1-1 (with E21 and E22) and NR1-3 (lacking E22, but containing E21) by approximately 2-fold, respectively (Fig. 1B, C). Formalin treatment did not significantly affect the expression pattern of the NR1 splice variants.

Expression of mRNA encoding NR2 subunits

Semi-nested hot start PCR was used to co-amplify the NR2A-D cDNA fragments in a manner reflecting the initial relative proportions of the four NR2A-D subunit specific mRNAs (Flint et al., 1997; Allgaier et al., 2001). Comparison of the chemiluminescence intensities for PCR products and cloned standards allowed the calculation of the relative percentages of subunits NR2A-D (Fig. 2). In untreated control animals, NR2B exhibited the highest expression, followed by decreasing proportions of NR2C, NR2A, and NR2D (Fig. 2C). Formalin treatment significantly increased the proportion of NR2A and decreased that of NR2C, accordingly (Fig. 2C).

Discussion

The effect of formalin injection on mRNA expression coding for NMDA receptor splice variants and subunits was investigated in rat lumbar spinal cord (L3-6) using RT-PCR (Allgaier et al., 1999, 2001). Previous in-situ hybridisation studies performed on *cervical* spinal cord of control rats demonstrated NR1-b mRNA expression in most neurones throughout all the laminae and at a distinctly higher level than NR1-a mRNA (Luque et al., 1994), whereas in *lumbar* spinal cord this expression pattern was reversed (Tölle et al., 1995). NR1-1 splice variants were almost exclusively detected in high abundance in *lumbar* neurones of laminae I-III (Tölle et al., 1995); their *cervical* expression has not yet been investigated. NR1-2 splice variants were abundantly found in *cervical* and *lumbar* spinal cord (Luque et al., 1994; Tölle et al., 1995). In *cervical* and *lumbar* tissue, NR1-4 splice variants were widely distributed and predominated over the NR1-3 variants which were particularly found in dorsal horn neurons.

The present RT-PCR study performed on lumbar spinal cord using primer pairs flanking E21 or E21/E22 corroborates the expression pattern of mRNAs coding for the C-terminal variants (i.e. NR1-1 to NR1-4) mentioned above. The observation that the expression of NR1-b splice variants predominated over NR1-a using primer pairs flanking E5 corresponds with the results of Luque et al. (1994) but is in contrast to those of Tölle et al. (1995) (see above). However, the reliability

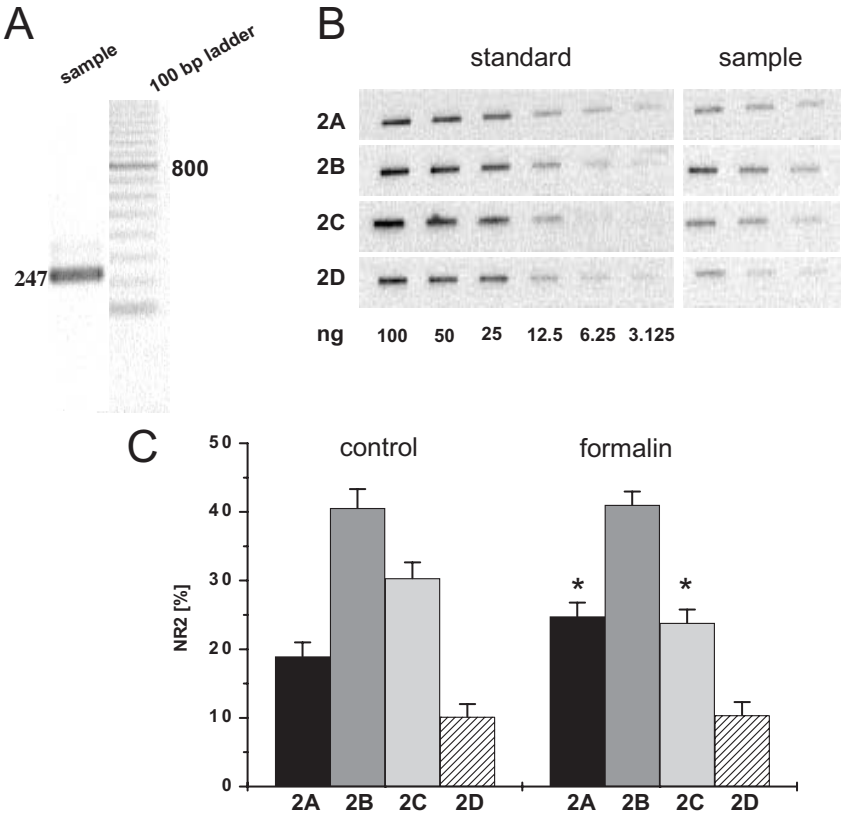

Fig. 2 A–C. NR2 subunit mRNA expression in spinal cord of control and formalin-treated rats. (**A**,**B**) Principles of RT-PCR and chemiluminescence detection of the NR2A-D fragment amplification products. Subsequent to formalin injection (5%) and preparation of segments L3–6 of the spinal cord, co-amplification of the 2A–D cDNA fragments was performed by semi-nested hot-start RT-PCR as described in Materials and methods. (**A**) Representative agarose gel electrophoresis (2%) of the ethidium bromide-stained 247 bp amplification products. (**B**) Chemiluminescence analysis of the PCR products by slot-blot hybridization. A serial dilution of each PCR product (sample; 1:2, 1:4, 1:8) was added onto four different nylon membranes each containing a serial dilution of the respective cloned NR2 subunit as standard (100–3.125 ng). The relative percentages of the subunits NR2A–D were calculated for each sample by comparing the intensity of signals for each PCR product and NR2 standard. (**C**) Expression pattern of mRNA encoding NR2A–D. Relative percentages of expression of each NR subunit were calculated from the corresponding total expression values (= 100%). Means ± SEM of 8 experiments from 4 animals are given for each group. The asterisks indicate significant differences vs. control animals (t-test, $p < 0.05$)

of the present data was supported by control experiments (data not shown) on rat neocortical and cerebellar cDNA preparations, in which the overall predominating splice variants of NR1 were those lacking and containing the 5′ inserts, respectively (Zhong et al., 1995). A high level of NR1-b expression would also match the general observation that the splice variants containing the N-terminal insert are expressed in relative abundance in cerebral structures associated with motor control (Laurie et al., 1995).

NR1 isoforms exhibit distinct functional properties. In particular, protons and polyamines act in a reciprocal manner on the N-terminal splice variants. Homomeric assemblies of NR1-a form receptors that exhibit higher apparent affinities for glutamate and NMDA, but distinctly smaller currents, and increased sensitivities to protons, spermine and Zn^{2+} compared to receptors containing NR1-b variants (Durand et al., 1993; Hollman et al., 1993). For example, at pH 7.3 protons inhibited NR function by about 50% accounting for the lower current amplitude of NR1-a homomers, while NR1-b homomers were fully active at this pH (Traynelis et al., 1995). Proton inhibition of NR function was also observed after co-expression of NR1-a with NR2A, NR2B, or NR2D but not in NR1-a/NR2C combinations, and was dramatically reduced in heteromeric NR containing NR1-b. Accordingly, the function of receptors containing NR1-a variants may be substantially affected by acidification of the extracellular fluid occurring under pathophysiological conditions such as inflammation. Also C-terminal splicing affects NR function and may play a pivotal

role in long-term regulation of receptor clustering (Chandler et al., 1998).

Glutamate released particularly from C-fibres contributes to formalin-induced pain responses by acting at NMDA receptors (Coderre et al., 1990). Accordingly, competitive NR antagonists as well as glycine B receptor site antagonists diminish the formalin-induced pain responses in a dose-dependent manner (Vaccarino et al., 1993; Chaplan et al., 1997). However, subsequent to formalin treatment the expression pattern of the NR1 splice variants was not changed significantly.

NR2 subunits are more restrictedly expressed than the NR1 subunit, and their expression pattern is differentially regulated during development (Monyer et al., 1994; Zhong et al., 1995; Vallano et al., 1998). For example, in cerebellum the NR2B mRNA level declined to undetectable levels during maturation, whereas NR2C mRNA was initially not detectable but subsequently increased to adult levels by postnatal day 28 (Zhong et al., 1995). Since the subunit composition of the NR specifies the single channel and kinetic properties, as well as the sensitivities to agonists, antagonists and cofactors, developmental changes in subunit expression are associated with changes in the receptor properties (Vallano et al., 1998).

Some conflicting results exist on the expression of NR2 subunits in the spinal cord of adult rats. In-situ hybridisation studies detected distinctly higher levels of NR2A mRNA compared to NR2B mRNA in cervical spinal cord (Luque et al., 1994) but failed to detect either NR2A or NR2B mRNA in lumbar sections (Tölle et al., 1993). In contrast, Western blot analysis using subunit specific antibodies demonstrated that levels of NR2A and NR2B protein were significantly higher in lumbar cord than in the cervical segment (Grossman et al., 2000). In rat lumbar spinal cord, NR2A immunoreactivity was widespread and observed in cell bodies throughout the dorsal and ventral horn, whereas NR2B immunoreactivity showed a more restricted distribution, with moderate staining of fibres in laminae I–II of the dorsal horn suggesting a presynaptic location on primary afferent fibres (Boyce et al., 1999). Yung (1998) corroborated the observation for NR2B, but was unable to detect any NR2A immunoreactivity in the dorsal horn of the rat. NR2C and NR2D subunits were detected only in low abundance in rat spinal cord (Tölle et al., 1993). NR2C mRNA expression appears to be restricted to certain cells of lamina II, whereas NR2D mRNA was distributed more widespread but at a low level (Tölle et al., 1993). It remains open whether technical reasons or variations in the rat strain used may account for the discrepancies on NR2 expression observed even with an identical methodological approach in particular on NR2A and NR2B expression (see above). The present RT-PCR study, in which the NR2A-D cDNA fragments were co-amplified in a manner reflecting the initial proportions of the 4 mRNAs specifically encoding subunits NR2A-D, demonstrates a predominant expression of NR2B mRNA, and a lower but distinct expression of each of the other 3 subunits. The distinct detection of NR subunits 2C and 2D may derive from the higher sensitivity of the RT-PCR approach in comparison to the in situ-hybridisation or immunocytochemical studies mentioned above.

In the present study performed on rats, formalin caused a significant increase and decrease in NR2A and NR2C mRNA expression, respectively, 6 hours after injection. In the same species, changes in NR2A expression were also observed in response to a standardized contusive spinal cord injury (SCI) (Grossman et al., 2000) by in situ hybridisation. NR2A mRNA was increased in thoracic ventral motor neurons (VMN) caudal to the injury site at 24 h after contusive SCI. One month after the injury, NR2A mRNA was elevated not only in thoracic motor neurons but also in lumbar motor neurons. The properties of NR are largely influenced by their subunit composition. For example, recombinant NR formed of NR1/NR2C or NR1/NR2D had several features that distinguished them from channels containing NR2A, such as higher affinities for glutamate, longer offset decay time constants, lower elementary conductances, and a reduced sensitivity to blockade by Mg^{2+} ions (Monyer et al., 1994; Vallano et al., 1998). However, in rat locus coeruleus neurones with a predominant NR2C mRNA expression NMDA induced larger currents than in cells lacking expression of this subunit (Allgaier et al., 2001).

In conclusion, the present data demonstrate for the first time changes in NR subunit expression subsequent to formalin injection. They presumably derive from an increase in glutamate release from C-fibre afferents which are intensely activated upon formalin administration (Coderre et al., 1990). Also in rat cultured cortical and cerebellar neurons a sustained increase in neuronal activity induced by elevated concentrations of extracellular K^+ was associated with an enhanced expression of mRNA coding for NR2A (Vallano et al., 1996; Mühlberg and Allgaier, 2000).

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