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The anti-craving compound acamprosate acts as a weak NMDA-receptor antagonist, but modulates NMDA-receptor subunit expression similar to memantine and MK-801

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Abstract

NMDA-receptor-mediated mechanisms may be crucial in addictive states, e.g. alcoholism, and provide a target for the novel anti-craving compound acamprosate. Here, the pharmacological effects of acamprosate on NMDA-receptors were studied using electrophysiological techniques in different cell lines in vitro. Additionally, a possible modulation of brain NMDA-receptor subunit expression was examined in vivo in rats, and compared to two effective non-competitive NMDA-receptor antagonists, memantine and MK-801. Electrophysiology in cultured hippocampal neurons (IC_{50} approx. 5.5 mM) and *Xenopus* oocytes (NR1-1a/NR2A assemblies: IC_{50} approx. 350 μM, NR1-1a/NR2B: IC_{50} approx. 250 μM) consistently revealed only a weak antagonism of acamprosate on native or recombinant NMDA-receptors. In HEK-293 cells, acamprosate showed almost no effect on NR1-1a/NR2A or NR1-1a/NR2B recombinants (IC_{50}s not calculated). Protein blotting demonstrated an up-regulation of NMDA-receptor subunits after acamprosate as well as after memantine or MK-801, in comparison to controls. After acamprosate, protein levels were increased in the cortex (NR1-3/1-4: 190±11% of controls) and hippocampus (NR1-1/1-2: 163±11%). The up-regulations observed after memantine (cortex, NR2B: 172±17%; hippocampus, NR1-1/1-2: 156±8%) or MK-801 (cortex, NR2B: 174±22%; hippocampus, NR1-1/1-2: 140±3%) were almost identical. No changes were detected in the brainstem. The present data indicate an extremely weak antagonism of NMDA-receptors by acamprosate. However, its ability to modulate the expression of NMDA-receptor subunits in specific brain regions — shared with the well established NMDA-antagonists memantine and MK-801 — may be of relevance for its therapeutic profile, especially considering the growing importance of NMDA-receptor plasticity in the research of ethanol addiction.

Keywords: Anti-craving compounds; Acamprosate; Memantine; MK-801; Ethanol; Alcohol; Protein expression; NMDA-receptor; Splice variants; Subunits

1. Introduction

In the past years there has been increasing evidence that glutamatergic mechanisms play a central role in the pathophysiology of alcoholism (Tsai and Coyle, 1998; Wickelgren, 1998). Conceivably, compounds which interact with glutamatergic signalling may therefore influence addictive symptoms such as the intense drug cravings addicts undergo during absence of e.g. ethanol (Lovinger and Zieglgänsberger, 1996). Due to its "anti-craving" properties the synthetic compound acamprosate (calcium-acetylhomotaurinate) recently has been clinically introduced as a new treatment for alcohol relapse (Sass et al., 1996; for review see Dahchour and DeWitte, 2000). Its effects are thought to involve primarily the modulation of glutamatergic pathways, especially NMDA-receptor-mediated signalling (Spanagel and Zieglgänsberger, 1997). A recent binding study in the rat suggested specific acamprosate binding to the spermi-

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dine-sensitive site (Naassila et al., 1998), which would allosterically modulate the NMDA-receptor in a complex manner (Johnson, 1996). Electrophysiological studies which tested acamprosate in rat brain slices (Zeise et al., 1993; Madamba et al., 1996; Berton et al., 1998) corroborated a modulation of glutamatergic neurotransmission, but the various findings appear inconsistent. In comparable concentrations acamprosate (0.1–1 mM) decreased NMDA-induced postsynaptic potentials in cortical neurons (Zeise et al., 1993), whereas in neurons of the hippocampal CA1 region and of the nucleus accumbens it enhanced NMDA-receptor-mediated responses (Madamba et al., 1996; Berton et al., 1998). The known differential subunit composition of NMDA-receptors in different brain areas (Laurie and Seeburg, 1994; Laurie et al., 1995) may account for this discrepancy of drug action (Spanagel and Zieglgänsberger, 1997), however, this hypothesis has not been tested.

Besides acamprosate, the non-competitive NMDA-receptor antagonist memantine (for review see Parsons et al., 1999) represents another substance which was recently proposed as a putative anti-craving and anti-relapse compound, abolishing relapse drinking in an animal model of alcoholism (Hölter et al., 1996) similar to acamprosate (Spanagel et al., 1996). Memantine and other non-competitive NMDA-receptor antagonists have also been shown to generalize the ethanol cue in rats (Shelton and Balster, 1994; Hundt et al., 1998) and produce dose-related ethanol-like subjective effects in detoxified alcoholics (Krystal et al., 1998). This may indicate that the decreasing effects of memantine on ethanol intake may be due to a substitution of the stimulus properties of ethanol at the NMDA-receptor. Together, the use of NMDA antagonists may provide promising new strategies in the treatment of alcoholism.

Glutamatergic pathways are widespread in the central nervous system and glutamate represents the major excitatory amino acid neurotransmitter. The NMDA subtype of glutamate receptor is not only important in mediating neuronal plasticity, but is also involved in multiple physiological and pathological functions including the pathomechanism of addiction (Spanagel and Zieglgänsberger, 1997; Parsons et al., 1999). Considering the presently limited understanding of the neuropharmacological mechanisms of acamprosate, a major objective of this study was to investigate further the electrophysiological properties of acamprosate on NMDA-receptor-mediated signalling. Little is known about the direct pharmacological effects of acamprosate on NMDA-receptor channels. In order to exclude methodological drawbacks which may be associated with brain slice preparations, e.g. possible indirect effects via other, uncontrolled mechanisms, the pharmacological effects of acamprosate were studied in vitro in cultured hippocampal neurons as well as on recombinant NMDA-receptors expressed in two different cell systems (HEK-293, *Xenopus laevis* oocytes). In addition, as dynamic expression changes of NMDA-receptor subunits may also play an important role in the pathomechanism of alcoholism (Follesa and Ticku, 1996a; Trevisan et al., 1994; Snell et al., 1996; Winkler et al., 1999; Wirkner et al., 1999), it was also examined whether acamprosate has the potential to regulate the expression level of NMDA-receptor subunits. Such possible modulatory effects were tested in selected brain regions (brainstem, hippocampus, cortex) in vivo in normal rats and compared to the well characterized uncompetitive NMDA-receptor antagonists memantine and MK-801.

2. Material and methods

2.1. Electrophysiology

2.1.1. Recombinant NMDA-receptors in HEK-293 cells

HEK-293 cells (human embryonic kidney cells; ATCC, CRL 1573) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Germany) with phenol red supplemented with 10% charcoal-stripped steroid-free foetal calf serum for reporter gene assays (Arizza et al., 1987) and 100 U/ml penicillin and 100 U/ml streptomycin. Three hours before transfection the medium was replaced by a glutamine-free medium. Transfections were performed using an electroporation system (Biotechnologies and Experimental Research, San Diego, CA, USA) after the determination of the optimal electric field strength (Chu et al., 1987). Cells, taken from a 100 mm dish at a density of 3×10^6, were then transfected with equimolar, 1:2 or 1:3 amounts of NR1-1a with either NR2A or NR2B (rat cDNAs; all constructs were obtained from P.H. Seeburg, University Heidelberg ZMBH, Heidelberg, Germany). In the expression vectors, the various receptor subunit cDNAs were under the control of the cytomegalovirus promotor and the sp6 promotor sequences. Following transfection, cells were grown in the presence of the NMDA-receptor antagonist D(−)-2-amino-5-phosphonopentanoic acid (AP5; 1 mM) to prevent cell death (Anegawa et al., 1995). In order to visualize transfected cells for electrophysiological patch-clamp experiments, transfections were performed in the presence of 5 μg reporter gene (green fluorescence protein, GFP; Heim et al., 1995). The total amount for these triple transfections was approximately 25 μg. Cells were replated on 35 mm dishes to a density of approximately 1×10^4 per dish to avoid cell agglomeration. Dishes were then incubated for 20 h in a humidified incubator in 5% CO_2 at 37°C.

Transfected cells were recorded in the whole-cell voltage-clamp configuration of the giga-seal technique under visual control using an inverted microscope (Zeiss, Jena, Germany). The cells were kept in a bath solution containing (in mM) 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 10 HEPES, 0.003 Glycine, 10 D-Glucose. The pH was adjusted to 7.4. Patch electrodes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) using a horizontal pipette puller (Zeitz-Instruments, Augsburg, Germany) to yield pipettes with a resistance of 3–6 MΩ. Pipettes were filled with a solution containing (in mM) 100 *N*-methyl-D-glutamine, 40 CsF, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 2 Mg-ATP. The pH was adjusted to 7.4. After the whole-cell configuration was established, the cells were lifted from the glass substrate and glutamate (1 mM) and acamprosate (Lipha, Lyon, France) were applied at the indicated concentrations using a fast superfusion device. A piezo translator-driven double-barrelled application pipette was used to expose the lifted cell either to glutamate-free or glutamate-containing solution for control experiments. A 2 s glutamate pulse was delivered every 60 s. Acamprosate was dissolved in aqua-bidest and diluted with bath solution to the desired concentration. Current signals were recorded at a holding potential of −50 mV with an EPC-9 amplifier (List, World Precision Instruments, Berlin, Germany) using Pulse+PulseFit (Heka 8.31, Heka Elektronik, Lambrecht, Germany) and IgorPro v.3.03 (Wavemetrics, Lake Oswego, Oregon) software on a MacIntoshII computer. Only results from stable cells were accepted for inclusion in the final analysis, i.e. showing at least 75% recovery of responses to glutamate following removal of acamprosate.

2.1.2. Recombinant NMDA-receptors in Xenopus oocytes

The rat cRNAs encoding the subunit NR1-1a and the subtypes NR2A/2B were obtained from JP Ruppersberg, University Tübingen, Germany. Mature female *Xenopus laevis* were anaesthetized in 0.2% tricaine on ice for 15 min prior to surgery. Oocytes were removed and incubated in 2 mg/ml collagenase (type II) in Ca^{2+}-free oocyte Ringer solution (in mM: 82.5 NaCl, 2 KCl, 2 $MgCl_2$, 5 HEPES, pH 7.5) for 30 min at room temperature and washed thoroughly with oocyte ringer 2 (OR-2; in mM: 100 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, pH 7.5). The remaining follicle cell layer was removed manually with fine forceps and the oocytes were kept in OR-2. The cRNA was dissolved in DEPC-treated, sterile distilled water. The NR1-1a subunit was mixed 1:1 with either NR2A or NR2B cRNA. Fifty nanoliters of each cRNA mixture were injected into the oocyte's cytoplasm using a Nanoliter Injector (List, World Precision Instruments, Berlin, Germany). The oocytes were incubated at 19°C in OR-2 for the following 3–6 days.

The electrophysiological responses were obtained using the standard two-electrode voltage-clamp method (GeneClamp 500 amplifier), 3–6 days after cRNA injection. The electrodes had a resistance between 0.2 and 1 MΩ and were filled with 3 M KCl. Recordings were made in a custom made chamber with 2–3 s exchange times. The bath solution was prepared Ca^{2+}-free, in order to avoid Ca^{2+}-induced Cl^- − currents (in mM: 100 NaCl, 2 KCl, 5 HEPES, 2 $BaCl_2$, pH 7.35). NMDA-receptors were activated by the manual co-application of 1 mM glutamate and 10 μM glycine for 30–40 s every 2–3 min to oocytes clamped at −70 mV.

2.1.3. Cultured hippocampal neurons

Hippocampi were obtained from rat embryos (E20 to E21) and transferred to Ca^{2+}- and Mg^{2+}-free Hank's buffered salt solution (Gibco, Germany) on ice. Cells were mechanically dissociated in 0.05% DNAase/0.3% ovomucoid (Sigma, Deisenhofen, Germany) following an 8 min pre-incubation with 0.66% trypsin/0.1% DNAase (Sigma). The dissociated cells were then centrifuged at 18*g* for 10 min, re-suspended in minimum essential medium (Gibco) and plated at a density of 150,000 cells cm^{-2} onto poly-DL-ornithine (Sigma)/laminin (Gibco)-precoated plastic Petri dishes (Peske, Germany). The cells were nourished with $NaHCO_3$/HEPES-buffered minimum essential medium supplemented with 5% foetal calf serum and 5% horse serum (Gibco) and incubated at 37°C with 5%CO_2 at 95% humidity. The medium was completely exchanged following inhibition of further glial mitosis with cytosine-β-D-arabinofuranoside (ARAC, 5 μM, Sigma) after about 5 days in vitro.

Patch-clamp recordings were made from these neurons after 12–15 days in vitro with polished glass electrodes (4–5 MΩ) in the whole-cell mode at room temperature (20–22°C) with the aid of an EPC-7 amplifier (List, World Precision Instruments, Berlin, Germany). Test substances were applied by switching channels of a custom made fast superfusion system with a common outflow (15–20 ms exchange times). The contents of the intracellular solution were as follows (in mM): 120 CsCl, 20 TEACl, 10 EGTA, 1 $MgCl_2$, 0.2 $CaCl_2$, 10 glucose, 2 ATP, 0.25 cAMP; pH was adjusted to 7.3 with CsOH or HCl. The extracellular solutions had the following basic composition (in mM): 140 NaCl, 3 KCl, 0.2 $CaCl_2$, 10 glucose, 10 HEPES, 4.5 sucrose, tetrodotoxin (TTX 3×10^{-4}). Glycine (1 μM) was present in all solutions sufficient to cause around 80–85% activation of $glycine_B$ receptors. NMDA-receptors were activated by application of 200 μM NMDA. Only results from stable cells were accepted for inclusion in the final analysis i.e. showing at least 75% recovery of responses to NMDA following removal of acamprosate.

2.1.4. Data analysis

In all electrophysiological experiments acamprosate was pre-incubated at each concentration before co-application with agonist. IC_{50}-values were calculated according to the logistic equation

$$y=\frac{100\%}{1+\left(\frac{x}{IC_{50}}\right)^{s}}$$

using the Grafit computer program (Erithacus Software, England) or IgorPro (Wavemetrics). All data presented are expressed as mean±SEM.

2.2. Protein expression experiments

2.2.1. Animal treatment

All animal experiments were conducted with the approval of the Institutional Animal Use and Care Committee. All animals (male Wistar rats, 250–300 g; Charles River, Sulzfeld, Germany) were kept under controlled lighting conditions (12:12 h light/dark cycle with the light phase commencing at 7.00 a.m.). They were housed four per cage in a colony room where temperature and humidity were kept constant at 21±1°C and 60±5%, respectively. All experimental animals were injected intraperitoneally (n=4 per experimental group) with the following compounds: saline (control group); acamprosate (200 mg/kg); double injection of acamprosate separated by a 12 h interval (2×200 mg/kg); memantine (10 mg/kg) or MK-801 (1 mg/kg). The doses used for the various compounds were selected on the basis of preliminary studies on mRNA expression (Putzke et al., 1997). Memantine was obtained from Merz and Co. (Frankfurt, Germany).

2.2.2. Brain tissue preparation

Six hours after injection (after the second injection in the case of the repeated acamprosate application) animals were decapitated and brains removed. Brains were rapidly dissected into hippocampus, fronto–parietal cortex and brainstem. Dissected brain tissues were immediately frozen on dry ice and kept at −80°C until used.

2.2.3. Western blotting

Each of the various brain regions was homogenized in HEPES buffer containing 1% NP40 and several proteinase inhibitors (based on Hope et al., 1994), and centrifuged to eliminate cell debris. The supernatant was used as total protein sample. Protein concentration was determined with the BioRad DC protein kit (BioRad, München, Germany). Protein samples of four animals per group and brain region investigated were pooled and 25 μg protein per lane were loaded on 9% SDS–PAGE and transferred to nitrocellulose (Protran BA85, 45 μm, Schleicher and Schüll, Dassel, Germany), using a Mini Transfer Cell (BioRad). The membranes were blocked with 5% BSA in TBS containing 0.1% Tween 20 (TBS-T) and incubated with the different primary antibodies overnight. Antibodies against NR1 (NR1-1/1-2; CHE Ab1516, Biozol, Eching, Germany), NR1 alternative C-terminus (NR1-3/1-4; UB 06-314, Biozol) and NR2B (CHE Ab1557P, Biozol) were employed. Incubation with the secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit antibody, Amersham Buchler, Braunschweig, Germany) lasted 2 h. All antibody incubations, washes and dilutions were performed in TBS-T. Antibody detection was performed with Amersham ECL Western blotting analysis system according to the manufacturer's protocol. ECL signal was exposed to Hyperfilm-ECL (Amersham Buchler). For control of protein amounts, samples (25 μg) were run on a 9% SDS–PAGE and the gels were stained with Coomassie Brilliant Blue. One gel was stained with Coomassie Blue and used to normalize the data and ensure equal loading on each well of the gel. To check for efficacy of protein transfer from the gel and to match Coomassie-stained bands with NR protein levels, reversible staining of the membrane was also done with Ponceau S after transfer to ensure homogeneity of transfer. The data were normalized to the amount of protein loaded in each lane of the Coomassie Blue stained gel that was run in parallel by measuring the intensity of the whole lane.

Unless otherwise stated all chemicals were obtained from Sigma (Deisenhofen, Germany). For each of the three brain regions investigated at least three blots were prepared per antibody which were analyzed and averaged for each region and antibody. Each Western blot comprised the control and the remaining four experimental groups. Blot autoradiographs were quantified by computer-assisted densitometry using the Optimas image analysis system (Optimas/BioScan, Edmonds, USA). The results are presented descriptively. All data are expressed as relative grey values and the values for the different groups were determined by setting the control group to 100% and calculating the relative percentages of the other groups. The data for the different groups were normalized to the amount of protein loaded in each lane in the Coomassie blue-stained gel that was run in parallel.

2.2.4. Terminology of NR1 subunits

Alternative splicing generates eight isoforms for the NR1 subfamily (for review see Zukin and Bennett, 1995), and arise from splicing at three exons: a 21 amino acid insert in the N-terminal (N1) and a 37 or 38 amino acid insert in the C-terminal (C1, C2, respectively). In this study, terminology with non-capitalized suffixes was used, i.e. NR1-1a. The combination of the antibodies employed against NR1 subunits detected either the NR1-1a/1-1b and NR1-2a/1-2b subunits (all containing the C2 insert) and which we summarized to NR1-1/1-2, or the NR1-3a/1-3b and NR1-4a/4b subunits (all without the C2 insert) which we summarized to NR1-3/1-4 (For more information see also: Danysz and Parsons, 1998). In the electrophysiological experiments with HEK-293 cells or *Xenopus* oocytes exclusively the NR1-1a subunit was transfected.

3. Results

3.1. Electrophysiology

3.1.1. NMDA-induced currents through recombinant NMDA-receptors expressed in HEK-293 cells

Glutamate (1 mM), applied in Mg^{2+}-free solution containing 10 μM glycine, evoked characteristic whole-cell currents at a holding potential of −50 mV in transiently transfected HEK-293 cells, which expressed heteromeric NMDA-receptor channels consisting of NR1-1a in combination with NR2A or NR2B, respectively. Responses obtained from NR2A-containing co-assemblies were stronger (approximately 400 pA) than currents obtained from NR2B-containing coassemblies (around 200 pA). The same results had also been reported on the single channel level (Chen et al., 1999). In contrast to NR1-1a/NR2B, NR1-1a/NR2A-mediated current kinetics were characterized by marked desensitization. Therefore, only steady-state plateau currents were analyzed to exclude possible variations in peak currents due to small differences in the speed of solution flow and thereby concentration clamp speed.

Acamprosate displayed a similar weak potency in antagonizing NMDA-induced currents in HEK-293 cells expressing NR1-1a/NR2A and NR1-1a/NR2B receptors. Since concentrations higher than 1 mM acamprosate caused poor recording conditions and even cell death, it was not possible to accomplish a complete dose–response curve. Therefore, IC_{50} values could not be accurately calculated for either, NR1-1a/NR2A and NR1-1a/NR2B receptors (Fig. 1). The kinetics of the currents were not affected by the drug.

3.1.2. NMDA-induced currents through recombinant NMDA-receptors expressed in Xenopus oocytes

The effects of acamprosate were also investigated for NMDA-induced currents through recombinant NMDA-receptors in expressed *Xenopus* oocytes. In these experiments, activation of the NMDA-receptors was routinely obtained by prolonged (30–40 s) exposure of the oocytes to the control medium containing glutamate (1 mM) and glycine (10 μM) every 2–3 min, with oocytes clamped at −70 mV. In oocytes expressing NR1-1a/NR2A or NR1-1a/NR2B receptors acamprosate exerted a similar weak potency in antagonizing NMDA-receptor inward currents (Fig. 2) compared to the measurements in HEK-293 cells. The IC_{50}-values for NR1-1a/NR2A and NR1-1a/NR2B were extrapolated to 344±154 and 234±189 μM, respectively. The effect of the drug was concentration-dependent, but similar to the experiments in HEK-293 cells, at 1 mM acamprosate recording conditions were also unsatisfying and prevented testing higher concentrations. It was, therefore, also not possible to determine more accurate IC_{50}-values. Acamprosate showed no effects on response kinetics. It has been suggested, that acamprosate binds to the polyamine site at NR2B subunits. To address this question, we tested, whether acamprosate (0.6–200 μM) might antagonize the potentiating effect of the polyamine spermine (100 μM) on NR1-1a/NR2B. Acamprosate did not reverse the spermine-induced increase in receptor currents at any concentration tested (n=4; data not shown), indicating a different binding site for both compounds.

Testing acamprosate as the Ca^{2+} salt also adds excess Ca^{2+} to the solutions (one Ca^{2+} ion for two acamprosate molecules). As *Xenopus* oocytes express an endogenous Ca^{2+}-activated Cl^- − current it seemed pertinent to perform control experiments with 100 μM Ca^{2+} alone. In 4 of the 8 cells tested, Ca^{2+} also decreased NR2B receptor currents and a pronounced effect of acamprosate was only seen in these four cells. Thus, it seems that even the effects of high concentrations of acamprosate on NMDA receptors are not due to acamproaste itself, but rather due to non-specific effects of Ca^{2+} ions added.

3.1.3. Antagonism of acamprosate in cultured hippocampal neurons

Acamprosate (100–1000 μM) reduced whole-cell current responses of cultured hippocampal cells to NMDA (200 μM) in a concentration-dependent manner in the continuous presence of glycine (1 μM). The extrapolated IC_{50}-values corresponded to 5.36±1.47 mM (Hill 0.76) and 3.75±1.52 mM (Hill 0.58) for peak and plateau, respectively (Fig. 3). The kinetics of the inward currents were not affected by acamprosate. At concentrations above 1 mM acamprosate again exerted unspecific effects, and, similar to the experiments in oocytes, more accurate IC_{50}-values also could not be calculated. Comparable results were obtained in neurons exposed to 100 mM ethanol for 48 h prior to recording: IC_{50}-values 3.35±0.07 mM (Hill 0.83) and 1.88±0.13 mM (Hill 0.49) for peak and plateau responses, respectively (data not shown). Binding studies revealed that acamprosate may act as a partial co-agonist at the NMDA-receptor (Naassila et al., 1998; al Qatari et al., 1998), so that low concentrations enhance activation when receptor activity is low, whereas higher concentrations are inhibitory to high levels of receptor activation. Therefore, we also tested whether acamprosate displays differential effects depending on the level of NMDA-receptor activation. Fig. 4 shows the dose response curve for different agonist concentrations (NMDA 3–1000 μM) with and without acamprosate (100 μM). Overall, the effect of acamprosate on NMDA-induced currents was inde-

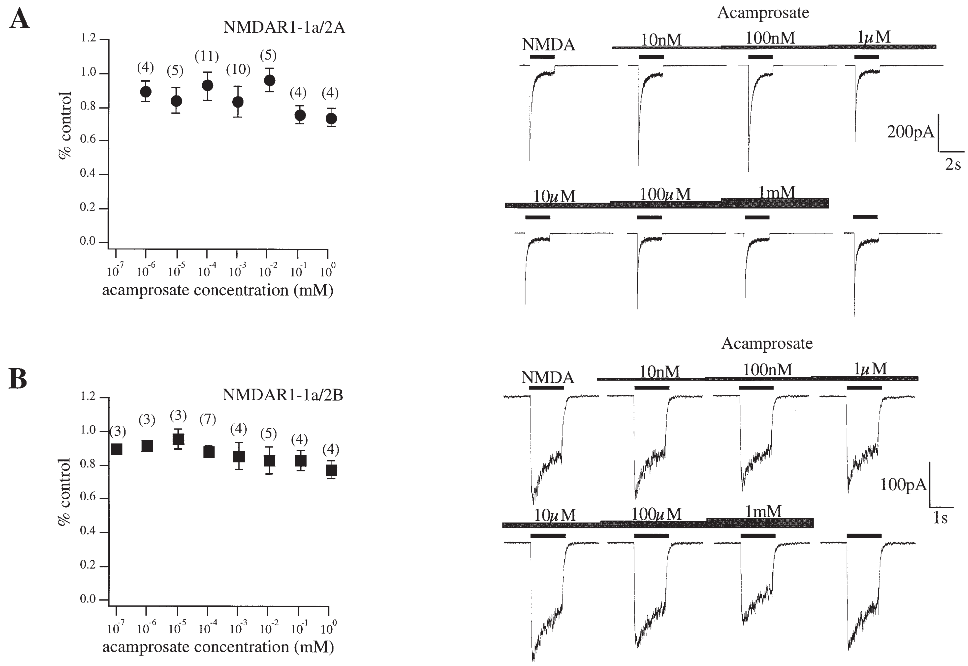

Fig. 1. Acamprosate displays only weak antagonist activity against NMDA-induced currents in HEK-293 cells expressing NR1-1a/NR2A or NR1-1a/NR2B receptors. NMDA receptor currents were induced by glutamate (1 mM)/glycine (10 μM) at −50 mV. Peak responses were normalized to control levels and plotted as means (±SEM) against acamprosate concentration. Estimation of IC_{50}-values and curve fitting were made according to the 4 parameter logistic equation. Number of concentrations tested indicated in brackets.

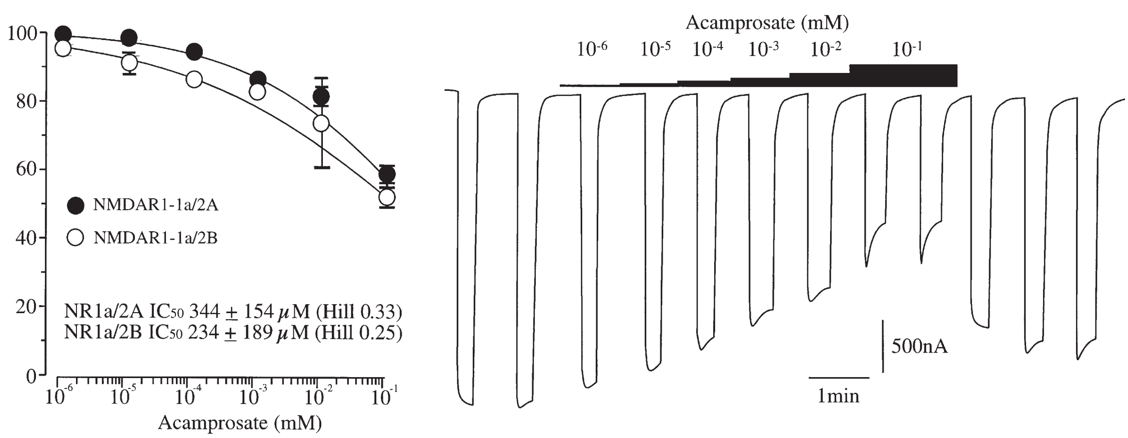

Fig. 2. Acamprosate is a weak antagonist against NMDA-induced currents in *Xenopus* oocytes expressing NR1-1a/2A or NR1-1a/2B receptors. Glutamate (1 mM)/glycine (10 μM) current responses were assessed at the end of 30–40 s agonist applications at −70 mV, with Ba^{2+} (1.5 mM) replacing Ca^{2+}. These responses were normalized to control levels and plotted as means (±SEM) against acamprosate concentration (n=6 per concentration). Estimation of IC_{50}-values and curve fitting were made according to the 4 parameter logistic equation.

pendent from the level of NMDA-receptor activation in the present experiments. EC_{50} of NMDA alone for plateau currents was 21.8±1.2 μM (Hill=1.70±0.12) and with acamprosate 21.5±1.4 μM (Hill=1.55±0.13). In accordance to the study of Priestley et al. (1994) we tested the expression of NMDA receptor subunits with the NR2B selective NMDA antagonist, ifenprodil, which discriminates in the present experiment two receptor populations, namely NR2A and NR2B, since the subunits NR2C and NR2D are not abundant in hippocampal pyramidal neurons (Monyer et al., 1994). Approximately 70% of NMDA-gated membrane current was antagonized with high affinity in cultured hippocampal neurones. These data suggest that in those cells we used for testing acamprosate, 70% of the population of NMDA receptors contained at least one NR2B subunit and 30% NR2A subunits (data not shown).

3.2. Protein expression experiments

3.2.1. Acamprosate

Both, the single dose of acamprosate (200 mg/kg) or the two times acamprosate application increased NR1-

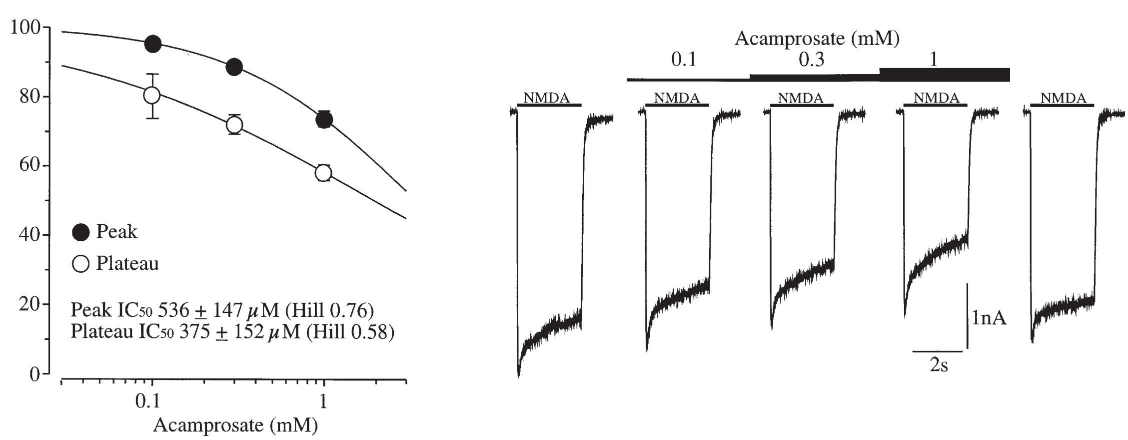

Fig. 3. Concentration-dependence of the blockade of NMDA-receptors by acamprosate in cultured hippocampal neurones. NMDA (200 μM) was applied for 2.5 s every 30 s in the continuous presence of glycine (1 μM) and various concentrations of acamprosate (100–1000 μM). Peak and steady-state (plateau) NMDA current responses were normalised to control levels and plotted as means (±SEM) against acamprosate concentration (n=8 per concentration). Estimation of IC_{50}-values and curve fitting were made according to the 4 parameter logistic equation. Original data for a single hippocampal neuron (Right). NMDA was applied as indicated by the bars. The left and right panels show control and recovery responses, respectively. The middle three panels show equilibrium responses in the continuous presence of acamprosate 100, 300 and 1000 μM, respectively.

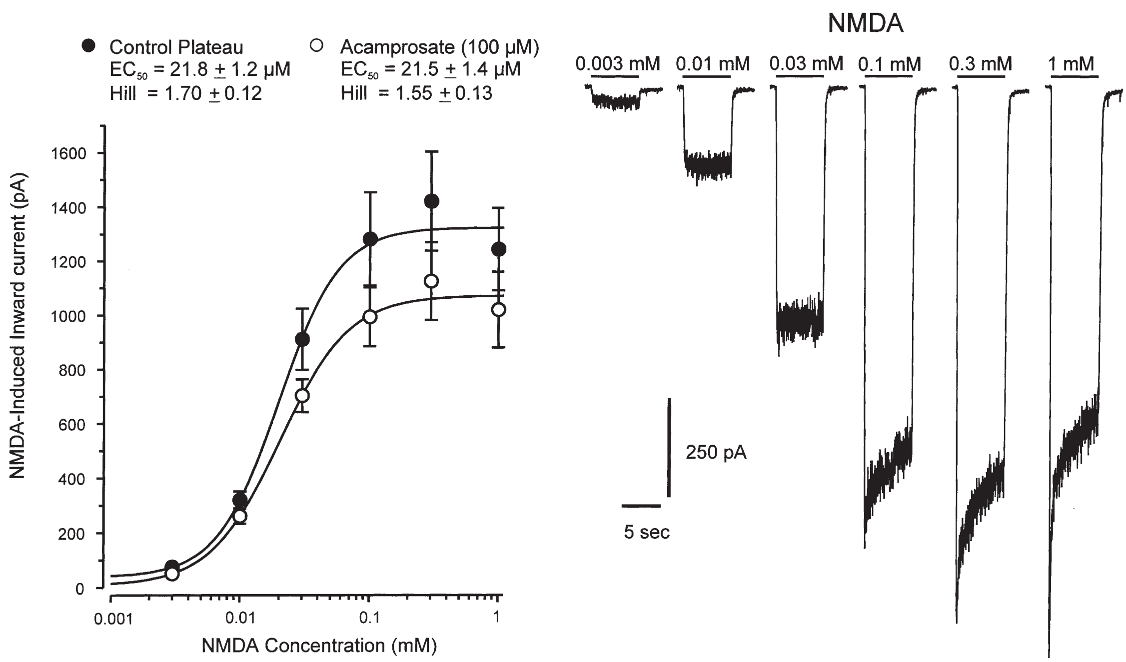

Fig. 4. The effect of acamprosate is not dependent on NMDA-receptor activation. Concentration dependence of NMDA-receptors by different NMDA concentrations in cultured hippocampal neurons. NMDA (3–1000 μM) was applied for 5 s every 30 s in the continuous presence of glycine (1 μM). The same experiment was performed with additional acamprosate (100 μM). Extrapolated EC_{50}-values being 21.8±1.2 μM (Hill 1.7) and 21.5±1.4 μM (Hill 1.55) for control and in the presence of acamprosate, respectively. Steady-state (plateau) NMDA current responses were normalised to maximum NMDA-response and plotted as means (± SEM) against NMDA concentration (n=8 per concentration). Estimation of EC_{50}-values and curve fitting were made according to the 4 parameter logistic equation. Original data for a single hippocampal neuron (right). NMDA was applied as indicated by the bars. The panels show equilibrium responses in the continuous presence of NMDA 3–1000 μM, respectively.

3/1-4 protein expression in the cortex to a comparable extent (179±13 or 190±11%, respectively, of saline-treated controls) (Fig. 5). NR2B protein expression level in the cortex was also up-regulated by the single dose of acamprosate (186±23%), and double application of acamprosate resulted in a similar (190±16%) increase of NR2B (Fig. 5). For NR1-1/1-2 protein expression in the cortex the values corresponded to 125±14% (single acamprosate) and 147±19% (two times acamprosate). In the hippocampus, no up-regulation of protein expression of the NMDA-receptor subunits investigated was observed after the single dose of acamprosate (NR1-1/1-2: 83±9%, NR1-3/1-4: 101±3%, NR2B: 105±9%) (Fig. 6). However, the repeated application of acamprosate

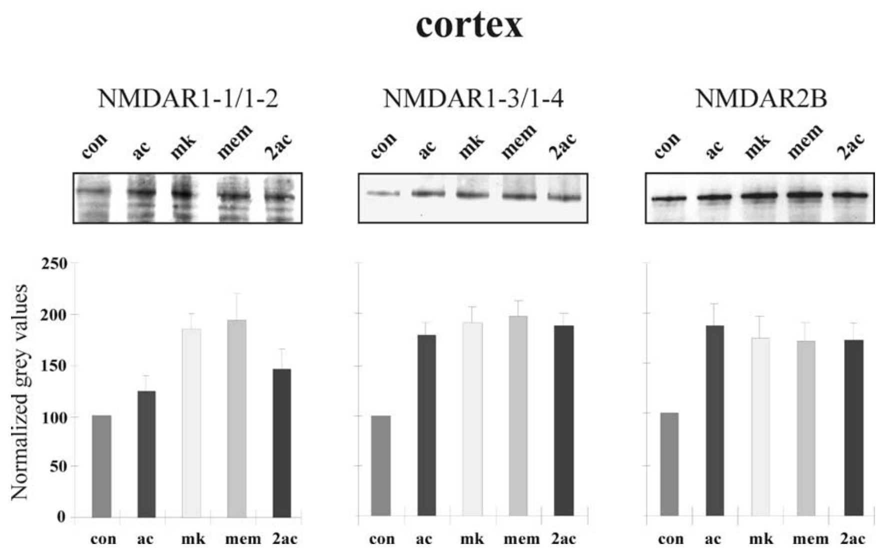

Fig. 5. Western blot analysis with NMDA-receptor antibodies (NR1-1/1-2, NR1-3/1-4, NR2B) in rat cortex. Four animals per group were pooled and 25 μg protein/lane was loaded on a SDS page. After immunoblotting, bands were analysed densiometrically. The control group was set at 100%, the other groups were calculated in percentage of control; *X*-axis of the diagram shows the experimental animal groups (con = saline-treated (i.p.) control animals; ac=200 mg/kg i.p. acamprosate; 2ac=2×200 mg/kg i.p. acamprosate; mem=10 mg/kg i.p. memantine; mk =1 mg/kg i.p. MK-801). *Y*-axis shows the optical density grey values in percentage.

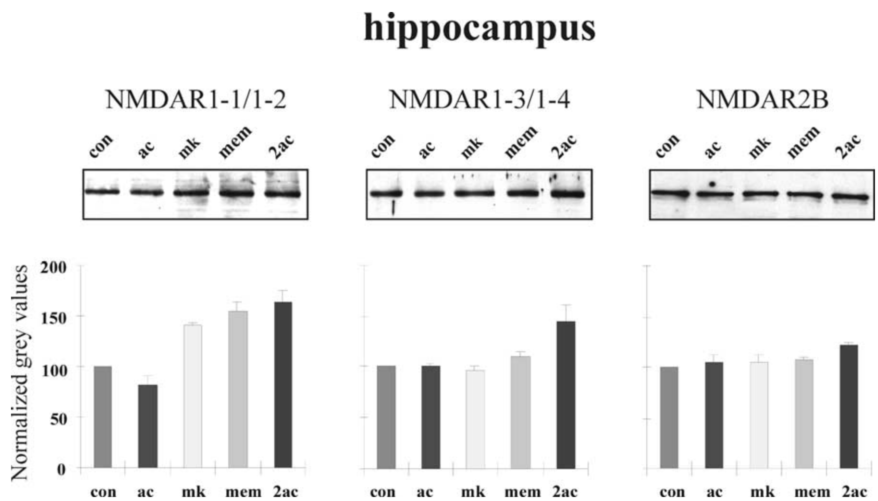

Fig. 6. Western blot analysis with NMDA-receptor antibodies (NR1-1/1-2, NR1-3/1-4, NR2B) in rat hippocampus. Four animals per group were pooled and 25 μg protein/lane was loaded on a SDS page. After immunoblotting, bands were analysed densitometrically. The control group was set at 100%, the other groups were calculated in percentage of control. *X*-axis of the diagram shows the experimental animal groups (con = saline-treated (i.p.) control animals; ac=200 mg/kg i.p. acamprosate; 2ac=2×200 mg/kg i.p. acamprosate; mem=10 mg/kg i.p. memantine; mk=1 mg/kg i.p. MK-801). *Y*-axis shows the optical density grey values in percentage.

increased protein expression of all subunits investigated (NR1-1/1-2: 163±11%, NR1-3/1-4: 145±17%, NR2B: 124±3%) (Fig. 6). In the brainstem no effect of acamprosate on NR1-3/1-4 protein expression could be detected (Fig. 7). NR1-1/1-2 protein was not detectable in this brain region and the lack of NR2B protein confirms its known absence in the brainstem (Hollmann and Heinemann, 1994).

3.2.2. Memantine and MK-801

Single administration of the non-competitive NMDA-receptor antagonists memantine or MK-801 showed nearly identical effects on the protein expression of the NMDA-receptor subunits investigated. Treatment with 10 mg/kg memantine increased NR1-1/1-2 (195±27%), NR1-3/1-4 (197±15%) and NR2B (172±17%) protein expression in the cortex (Fig. 5), and NR1-1/1-2

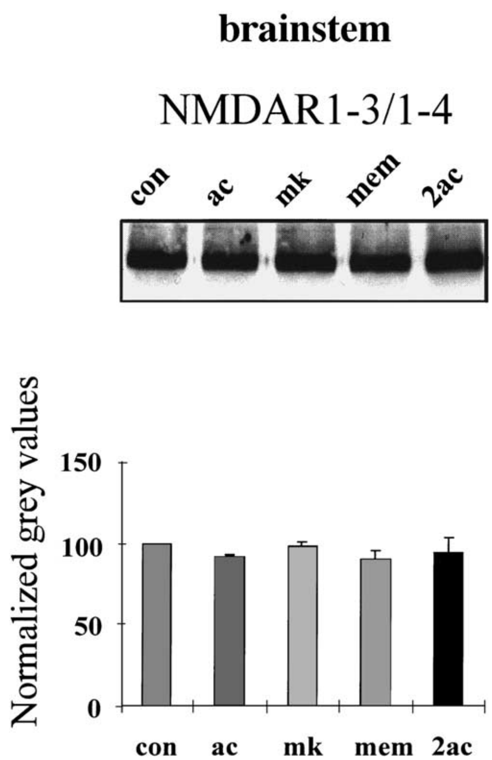

Fig. 7. Western blot analysis with NR1-3/1-4 antibodies in rat brainstem (no specific signal was observed for NR1-1/1-2 and NR2B in the brainstem). Four animals per group were pooled and 25 µg protein/lane was loaded on a SDS page. After immunoblotting, bands were analysed densitometrically. The control group was set at 100%, the other groups were calculated in percentage of control. *X*-axis of the diagram shows the experimental animal groups (con = saline-treated (i.p.) control animals; ac=200 mg/kg i.p. acamprosate; 2ac=2×200 mg/kg i.p. acamprosate; mem=10 mg/kg i.p. memantine; mk=1 mg/kg i.p. MK-801). *Y*-axis shows the optical density grey values in percentage.

(156±8%) in the hippocampus (Fig. 6). Application of 1 mg/kg MK-801 increased NR1-1/1-2 (186±14%), NR1-3/1-4 (193±14%) and NR2B (174±22%) protein expression in the cortex (Fig. 5), and NR1-1/1-2 (140±3%) levels in the hippocampus (Fig. 6). Similar to acamprosate treatment, no changes in subunit expression could be detected in the brainstem following MK-801 or memantine injections (Fig. 7).

4. Discussion

Patch-clamp studies performed in rat cultured hippocampal neurons and on recombinant NMDA-receptors expressed in HEK-293 cells or *Xenopus* oocytes consistently demonstrated only a very weak NMDA-receptor antagonist activity for acamprosate. Interestingly, additional in vivo experiments provided strong evidence for a modulatory effect of acamprosate on NMDA-receptor subunit expression in selected brain regions investigated. Acute acamprosate application increased the expression of specific NMDA-receptor subunits and such up-regulation was also observed for the well established NMDA antagonists memantine and MK-801. Thus, such modulatory potential of acamprosate on NMDA-receptor subunit expression — shared with known NMDA antagonists — rather then a direct pharmacological antagonism may be of important relevance for its mode of action.

4.1. Electrophysiology

Earlier studies in rat brain slices showed that acamprosate reduced the excitation induced by excitatory amino acids in cortical neurons (Zeise et al., 1993), but enhanced NMDA-receptor-mediated neurotransmission in hippocampal and nucleus accumbens neurons (Madamba et al., 1996; Berton et al., 1998). In the present study, recombinant NMDA-receptor currents, however, were antagonized only very weakly. This weak inhibition was very comparable in the different cell systems tested including the cultured hippocampal cells which strongly supports a general poor antagonism of acamprosate on NMDA-receptor complexes. It might be argued that the discrepancies between previous findings could have been due to different methodological approaches and/or differential subunit composition of NMDA-receptors in the brain regions examined (Laurie and Seeburg, 1994; Laurie et al., 1995; Spanagel and Zieglgänsberger, 1997). However, this is not supported by the present and a recent study (Popp and Lovinger, 2000) since acamprosate affected the currents through the physiologically distinct NR1-1a/2A and NR1-1a/2B co-assemblies in *Xenopus* oocytes or HEK-293 cells, as well as the currents in cultured hippocampal neurons with weak potency.

It has been discussed that variation in the endogenous polyamine concentration in different brain regions, due to a suggested polyamine concentration-dependency of acamprosate effects (al Qatari et al., 1998), might explain the inconsistent results mentioned above. In the present study, however, acamprosate did not reverse the positive modulatory effects of spermine on NR1-1a/NR2B receptors, indicating that acamprosate does not bind to the polyamine site.

The opposing actions of acamprosate in the aforementioned electrophysiological studies could also be concentration-dependent. Thus, in rat cortical membranes higher concentrations of acamprosate inhibited [^{3}H]dizocilpine (MK-801) binding, whereas at lower concentrations (<100 µM) [^{3}H]dizocilpine binding was enhanced (al Qatari et al., 1998), which points to a concentration-dependent biphasic acamprosate effect. However, in the present study employing various acamprosate concentrations in a similar range to the above-mentioned report, no concentration-dependent (biphasic) effects could be detected in the different cell systems. Yet another explanation may be proposed on the basis that acamprosate effects depend on the level of NMDA-receptor activation such, that at submaximal activation acamprosate acts as an enhancer, whereas at maximal activation acamprosate reduces NMDA-receptor activation (al Qatari et al., 1998; Naassila et al., 1998). The presently used agonist and co-agonist concentrations were sufficient to cause approximately 80–85% activation. Since acamprosate acted only as an antagonist in the present cell systems, receptor activation might have been too high to detect enhancing effects of acamprosate. However, this also seems unlikely, since using various NMDA concentrations revealed that the effect of acamprosate was entirely independent on NMDA-receptor activation (Fig. 4). Overall, acamprosate displayed dose-dependent but only weak antagonistic effects on recombinant NMDA-receptors in HEK-293 cells, *Xenopus* oocytes and cultured hippocampal neurons. This was observed for various NMDA-receptor co-assemblies and was not related to the level of NMDA-receptor activation.

The fact that acamprosate antagonized currents in NR2B receptors expressed in *Xenopus* oocytes only when Ca^{2+} alone also had an effect indicates that even the very weak antagonism seen with acamprosate was actually rather mediated via the Ca^{2+} in this salt. This effect was probably mediated via changes in Ca^{2+}-activated Cl^- − currents. A similar problem is less likely to dramatically disturb HEK-293 cells or native receptors in hippocampal neurones although Ca^{2+}-sensitive desensitization may indeed become somewhat more prominent. Thus, the extremely weak effects of acamprosate seen in HEK-293 cells and native receptors likely more closely reflects potency of acamprosate per se at NMDA receptors. These results might also explain that in some previous experiments acamprosate exhibited allosteric interactions with spermine and some experiments it did not (Naassila et al., 1998; Popp and Lovinger, 2000). The apparent interaction of acamprosate and spermine may depend on the sensitivity of the tested neurons to Ca^{2+} and therefore could be reduced to a Ca^{2+}-effect alone.

4.2. Protein expression experiments

Acute administration of acamprosate changed the protein expression of NMDA-receptor subunits in selected brain regions, similar to the non-competitive NMDA-receptor antagonists memantine and MK-801. Up-regulations after the single dose of acamprosate were most pronounced in the cortex (particularly of NR1-3/1-4 and NR2B). The double application produced very similar expression levels with an additional elevation in hippocampal NR1-1/1-2, NR1-3/1-4 and NR2B expression. Although the antibody used did not differentiate between NR1-3 and NR1-4, this most probably reflects an increase of NR1-4, since NR1-3 mRNA is only barely detectable in cortex and hippocampus (Laurie and Seeburg, 1994), and in preliminary experiments also no changes of NR1-3 mRNA were detected after acamprosate (Putzke et al., 1997). These studies at the mRNA level also indicated that the increases were more pronounced for NR1-1 compared to NR1-2 after acamprosate (Putzke et al., 1997), which suggests that the increases at the protein level, detected by the antibody against NR1-1/1-2, most probably reflect an increase in NR1-1 protein (see also Winkler et al., 1999). Single administration of MK-801 also induced an up-regulation of distinct NMDA-receptor subunits in cortex and hippocampus. In the cortex the alterations of NR1-3/1-4 and NR2B expression resembled the acamprosate-induced changes, with a more pronounced elevation in NR1-1/1-2 levels. In the hippocampus, however, the MK-801-induced changes appeared to show differential effects. The increase in NR1-1/1-2 expression may still be in line with the rise induced by the double injection of acamprosate, in contrast, no changes in NR1-3/1-4 and NR2B levels occurred. It cannot, however, be excluded that a higher or repeated dose of MK-801 would also regulate these latter subunits. Interestingly, both memantine and MK-801 showed almost identical modulation of NMDA-receptor subunit expression, which points to a compound-specific effect for non-competitive NMDA-receptor antagonists. In the brainstem, no changes were detected for NR1-3/1-4 expression after any of the compounds, corroborating a region-specific modulation of NMDA-receptor subunits in the brain (note that in the brainstem no specific signal was observed for NR1-1/1-2 and NR2B, which agrees with the known absence of NR2B mRNA (Hollmann and Heinemann, 1994)). Together, the present protein blotting data strongly suggest that NMDA-receptor subunit protein expression is rapidly, substance- and region-dependently increased after acute exposure to acamprosate or non-competitive NMDA-receptor antagonists. This also extends previous reports of a concentration- and time-dependent up-regulation of neuronal NMDA-receptor subunits after administration of NMDA antagonists in vitro (Williams et al., 1992; Follesa and Ticku, 1996b) and in vivo (Wilson et al., 1998). The similarities between acamprosate and known NMDA-receptor antagonists indicate common secondary effects on NMDA-receptor expression.

There may appear an important caveat in the interpretation of the data. In HEK-293 cells and in *Xenopus* oocytes acamprosate was tested on NMDA-receptors assembled exclusively with the NR1-1a subunit, whereas the specific antibody used detects all NR1-1/2 subunits (NR1-1a/b, NR1-2a/b). Therefore, the up-regulation in NMDA-receptor protein after acamprosate might also be explained by acamprosate possibly acting as a strong antagonist on NMDA-receptors assembled with other NR1-1/2 subunits, e.g. NR1-1b. This, however, was not investigated in the present patch-clamp experiments. But recent studies showed that in mature rats the NR1-1,2,3,4a subunits exhibit high expression levels in the hippocampus, whereas NR1-1,2,3,4b subunits predominate at early postnatal stages (Paupard et al., 1997). Furthermore, mRNA for double splice variants in the C1/C2 region, namely NR1-1a/1-1b, are more concentrated in rostral brain structures such as cortex and hippocampus (Laurie and Seeburg, 1994; Paupard et al., 1997). This indicates that the changes detected by the antibody against the NR1-1/2 subunits mainly reflect the NR1-1a subunit, also employed in the present electrophysiological studies. In addition, patch-clamp experiments performed in cultured hippocampal neurons (supposedly containing various kinds of NR1-1/2 subunits) revealed the same weak antagonism.

4.3. Possible pharmacological profile of acamprosate

An up-regulation of specific NMDA-receptor subunits contributes to functional changes of NMDA-receptors and thus most probably to functional changes in the brain sites involved. In vitro studies described NR1 splice variant-dependent functional differences (Hollmann and Heinemann, 1994), e.g. NR1-4 displays a lower agonist affinity and increased potentiation by PKC (Durand et al., 1993). NR1-4-containing NMDA-receptors are also more sensitive to ethanol (Criswell et al., 1998; Winkler et al., 1999). This and the present modulation of NR1-3/1-4 by acamprosate may point to the particular importance of this subunit in pathomechanisms of alcoholism. The central role of glutamatergic mechanisms in alcoholism became increasingly evident in the past years. Ethanol alters the synaptic action of glutamate especially by inhibition of the NMDA-receptor (Tsai and Coyle, 1998; Wirkner et al., 1999). Moreover, chronic ethanol increases NR1 splice variants and NR2 subunits (Follesa and Ticku, 1996a; Trevisan et al., 1994; Snell et al., 1996; Winkler et al., 1999). Thus, it may be proposed that the modulation of NMDA-receptor subunits by acamprosate and possibly also memantine is importantly involved in their therapeutical effects. In this study, both compounds showed an up-regulation of NMDA-receptor splice variants and subunits. Acamprosate produced these pronounced and specific changes despite the fact that it only acts (in contrast to memantine) as a very weak antagonist. This may indicate a novel pharmacological strategy which is distinct from any direct NMDA receptor antagonist actions of acamprosate, but rather targets neuronal plasticity involved in addiction by a different mechanism.

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References

Anegawa, N.J., Lynch, D.R., Verdoorn, T.A., Pritchett, D.B., 1995. Transfection of *N*-methyl-D-aspartate receptors in a nonneuronal cell line leads to cell death. Journal of Neurochemistry 64, 2004–2012.

Arizza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E., Evans, R.M., 1987. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237, 268–275.

Berton, F., Francesconi, W.F., Madamba, S.G., Zieglgänsberger, W., Siggins, G.R., 1998. Acamprosate enhances *N*-methyl-D-aspartate receptor-mediated neurotransmission but inhibits presynaptic GABA (B) receptors in nucleus accumbens neurons. Alcoholism Clinical and Experimental Research 22, 183–191.

Chen, N., Luo, T., Raymond, L.A., 1999. Subtype-dependence of NMDA-receptor channel open probability. Journal of Neuroscience 19, 6844–6854.

Chu, G., Hayakawa, H., Berg, P., 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Research 15, 1311–1326.

Criswell, H.E., Ming, Z., McCown, T.J., Rudolph, J.G., Crews, F.T., Breese, G.R., 1998. Influence of NMDA NR-1 splice variants on ethanol sensitivity. Alcoholism Clinical and Experimental Research 22, 6A.

Dahchour, A., DeWitte, P., 2000. Ethanol and amino acids in the central nervous system: assessment of the pharmacological actions of acamprosate. Progress in Neurobiology 60, 343–362.

Danysz, W., Parsons, C.G., 1998. Glycine and *N*-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacological Reviews 50, 597–664.

Durand, G.M., Bennett, M.V.L., Zukin, R.S., 1993. Splice variants of the *N*-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. Proceedings of the National Academy of Sciences of the United States of America 90, 6731–6735.

Follesa, P., Ticku, M.K., 1996a. Chronic ethanol-mediated upregulation of the *N*-methyl-D- aspartate receptor polypeptide subunits in mouse cortical neurons in culture. Journal of Biological Chemistry 271, 13297–13299.

Follesa, P., Ticku, M.K., 1996b. NMDA-receptor upregulation: Molecular studies in cultured mouse cortical neurons after chronic antagonist exposure. Journal of Neuroscience 16, 2172–2178.

Heim, R., Cubitt, A.B., Tsien, R.Y., 1995. Improved green fluorescence. Nature 373, 663–664.

Hölter, S.M., Danysz, W., Spanagel, R., 1996. Evidence for alcohol anti-craving properties of memantine. European Journal of Pharmacology 314, R1–2.

Hollmann, M., Heinemann, S., 1994. Cloned glutamate receptors. Annual Review of Neuroscience 17, 31–108.

Hope, B.T., Nye, H.E., Kelz, M.B., Self, D.W., Iadarola, M.J., Nakabeppu, Y., Duman, R.S., Nestler, E.J., 1994. Induction of a long-lasting AP-1 complex composed of altered fos-like proteins in brain by chronic cocaine and other chronic treatments. Neuron 13, 1235–1244.

Hundt, W., Danysz, W., Hölter, S.M., Spanagel, R., 1998. Ethanol and *N*-methyl-D-aspartate receptor complex interactions: a detailed drug discrimination study in the rat. Psychopharmacology 135, 44–51.

Johnson, T.D., 1996. Modulation of channel function by polyamines. Trends in Pharmacological Sciences 17, 22–27.

Krystal, J.H., Petrakis, I.L., Webb, E., Cooney, N.L., Karper, L.P., Namanworth, S., Stetson, P., Trevisan, L.A., Charney, D.S., 1998. Dose-related ethanol-like effects on the NMDA antagonist, ketamine, in recently detoxified alcoholics. Archives of General Psychiatry 55, 354–360.

Laurie, D.J., Seeburg, P.H., 1994. Regional and developmental heterogeneity in splicing of the rat brain NR1 mRNA. Journal of Neuroscience 14, 3180–3194.

Laurie, D.J., Putzke, J., Zieglgänsberger, W., Seeburg, P.H., Tölle, T.R., 1995. The distribution of splice variants of the NR1 subunit mRNA in adult rat brain. Molecular Brain Research 32, 94–108.

Lovinger, D.M., Zieglgänsberger, W., 1996. Interactions between ethanol and agents that act on the NMDA-type glutamate receptor. Alcoholism Clinical and Experimental Research 20 (Suppl.), 187A–191A.

Madamba, S.G., Schweitzer, P., Zieglgänsberger, W., Siggins, G.R., 1996. Acamprosate (calciumacetylhomotaurinate) enhances the NMDA component of excitatory neurotransmission in rat hippocampal CA1 neurons in vitro. Alcoholism Clinical and Experimental Research 20, 651–658.

Monyer, H., Burnashev, N., Laurie, D.J., Sakmann, B., Seeburg, P.H., 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12, 529–540.

Naassila, M., Hammoumi, S., Legrand, E., Durbin, P., Daoust, M., 1998. Mechanism of action of acamprosate. Part I. Characterization of spermidine-sensitive acamprosate binding site in rat brain. Alcoholism Clinical and Experimental Research 22, 802–809.

Parsons, C.G., Danysz, W., Quack, G., 1999. Memantine is a clinically well tolerated *N*-methyl-D-aspartate (NMDA) receptor antagonist — a review of preclinical data. Neuropharmacology 38, 735–767.

Paupard, M.C., Friedman, L.K., Zukin, R.S., 1997. Developmental regulation and cell-specific expression of *N*-methyl-D-aspartate receptor splice variants in rat hippocampus. Neuroscience 79, 399–409.

Popp, L.P., Lovinger, D.M., 2000. Interaction of acamprosate with ethanol and spermine on NMDA receptors in primary cultured neurons. European Journal of Pharmacology 394, 221–231.

Putzke, J., Mahal, B., Schadrack, J., Spanagel, R., Zieglgänsberger, W., 1997. NMDA-receptor antagonists influence the expression of NMDA-receptor subunits in the rat CNS. Pharmacopsychiatry 30, 208.

al Qatari, M., Bouchenafa, O., Littleton, J., 1998. Mechanism of action of acamprosate. PartII. Ethanol dependence modifies effects of acamprosate on NMDA-receptor binding in membranes from rat cerebral cortex. Alcoholism Clinical and Experimental Research 22, 810–814.

Sass, H., Soyka, M., Mann, K., Zieglgänsberger, W., 1996. Relapse prevention by acamprosate: results from a placebo controlled study in alcohol dependence. Archives of General Psychiatry 53, 673–680.

Shelton, K.L., Balster, R.L., 1994. Ethanol drug discrimination in rats: substitution with GABA agonists and NMDA antagonists. Behavioural Pharmacology 5, 441–450.

Snell, L.D., Nunley, K.R., Lickteig, R.L., Browning, M.D., Tabakoff, B., Hoffman, P.L., 1996. Regional and subunit specific changes in NMDA-receptor mRNA and immunoreactivity in mouse brain following chronic ethanol ingestion. Molecular Brain Research 40, 71–78.

Spanagel, R., Hölter, S.M., Allingham, K., Landgraf, R., Zieglgänsberger, W., 1996. Acamprosate and alcohol: I. Effects on alcohol intake following alcohol deprivation in the rat. European Journal of Pharmacology 305, 39–44.

Spanagel, R., Zieglgänsberger, W., 1997. Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes. Trends in Pharmacological Sciences 18, 54–59.

Tsai, G., Coyle, J.T., 1998. The role of glutamatergic neurotransmission in the patho-physiology of alcoholism. Annual Review of Medicine 49, 173–184.

Trevisan, L., Fitzgerald, L.W., Brose, N., Gasic, G.P., Heinemann, S.F., Dumand, R.S., Nestler, E.J., 1994. Chronic ingestion of ethanol up-regulates NR1 receptor subunit immuno-reactivity in rat hippocampus. Journal of Neurochemistry 62, 1635–1638.

Wickelgren, I., 1998. Teaching the brain to take drugs. Science 280, 2045–2047.

Williams, K., Dichter, M.A., Molinoff, P.B., 1992. Upregulation of *N*-mehyl-D-aspartate receptors on cultured cortical neurons after exposure to antagonists. Molecular Pharmacology 42, 147–151.

Winkler, A., Mahal, B., Kiianmaa, K., Zieglgänsberger, W., Spanagel, R., 1999. Effects of chronic alcohol consumption on the expression of different NR1 splice variants in the brain of AA and ANA lines of rats. Molecular Brain Research 72, 166–175.

Wilson, M.A., Kinsman, S.L., Johnston, M.V., 1998. Expression of NMDA-receptor subunit mRNA after MK-801 treatment in neonatal rats. Brain Research and Development Brain Res 109, 211–220.

Wirkner, K., Poelchen, W., Koles, L., Muhlberg, K., Scheibler, P., Allgaier, C., Illes, P., 1999. Ethanol-induced inhibition of NMDA-receptor channels. Neurochemistry International 35, 153–162.

Zeise, M.L., Kasparov, S., Capogna, M., Zieglgänsberger, W., 1993. Acamprosate (calciumacetylhomotaurinate) decreases postsynaptic potentials in the rat neocortex: possible involvement of excitatory amino acid receptors. European Journal of Pharmacology 231, 47–52.

Zukin, R.S., Bennett, M.V., 1995. Alternatively spliced isoforms of the NRI receptor subunit. Trends in Neurosciences 18, 306–313.