

Expression of *N*-methyl-D-aspartate (NMDA) receptor subunits and splice variants in an animal model of long-term voluntary alcohol self-administration

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Abstract

Long-term, free-choice, alcohol self-administration with repeated alcohol deprivation phases is known to enhance *N*-methyl-D-aspartate (NMDA) receptor activity. We hypothesized that this might not only reflect an increase in NMDA receptor density, but that differential transcriptional regulation and alternative splicing of the various subunits comprising the NMDA receptor may lead to changes in receptor composition and subsequent function. We, therefore, aimed to further investigate this effect in various brain regions. The relative mRNA expression of exon 5 inclusion/exclusion variants of the NR1 subunit, and the relative expression of NR2A, NR2B and NR2C subunits was examined in rats subjected to long-term free-choice, alcohol self-administration with repeated alcohol deprivation phases. We observed a relative decrease of the NR2C/NR2A mRNA ratio and an increase of NR1 splice variants including exon 5 (NR1 + E5) in the striatum but not in the cortex, hippocampus or cerebellum in the experimental group. Our results demonstrate that long-term voluntary alcohol self-administration, affects the regulation of genes encoding the various subunits and splice variants of the NMDA receptor in a brain regional-specific manner.

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1. Introduction

In order to study the molecular mechanisms of alcohol addiction, appropriate animal models are warranted. Although current animal models are generally unable to incorporate all aspects and criteria of alcohol dependence seen in humans, they can at least reflect some of the criteria given in the fourth edition of *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) of the American Psychiatric Association (1994). Here we used a rat model of long-term, free-choice, alcohol self-administration with repeated alcohol deprivation phases (Spanagel and Holter, 1999; Sanchis-Segura and Spanagel, 2006). These animals show

certain characteristics: (i) they exhibit relapse-like drinking behavior (=alcohol deprivation effect/ADE), (ii) they have a high, even compulsive motivation to consume alcohol during an ADE, (iii) they show tolerance to some effects of alcohol and have mild symptoms of physical withdrawal during the onset of abstinence, and (iv) during abstinence they exhibit psychological withdrawal symptoms consisting of enhanced anxiety-related behaviour and hyper-reactivity to stressful stimuli (Spanagel and Holter, 1999; Sanchis-Segura and Spanagel, 2006). In summary, these behavioral characteristics are very similar to those observed in alcohol dependent patients and therefore this animal model can be used for studying molecular mechanisms of alcohol addiction.

The *N*-methyl-D-aspartate (NMDA) receptor is a ligand-gated ion channel complex which belongs to the family of glutamate receptors. The receptor contains distinct ligand recognition sites sustaining both Na⁺ and Ca²⁺ currents and is essentially involved in learning and memory and is implicated

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in multiple neurological and psychiatric disorders (Waxman and Lynch, 2005; Nakazawa et al., 2004). Moreover, NMDA receptors are involved in mediating acute and chronic central nervous system (CNS) effects of ethanol (Lovinger et al., 1989; Tsai et al., 1995; Hoffman, 2003; Krystal et al., 2003; Vengeliene et al., *in press*). The NMDA receptor is thought to consist of a pentameric assembly of two NR1 subunits and three NR2 subunits, of which there are four distinct subtypes: NR2A, NR2B, NR2C, NR2D. Eight possible splice variants of NR1 exist, deriving from mRNA combinations including or excluding exon 5 and/or two 3' optional exons (exon 21, exon 22) (Nakanishi et al., 1992; Zukin and Bennett, 1995). Each of the respective NR1 splice variants and NR2 subunits confer distinct characteristics to the entire NMDA receptor complex (Chu et al., 1995). The different subunits/splice variants possess (i) specific ligand binding sites leading to variations in functional properties and affinities towards agonists and antagonists (Kutsuwada et al., 1992; Monyer et al., 1992) and (ii) differentially affect downstream signaling pathways (Bradley et al., 2006). In respect to ethanol, recombinant receptors display a range of ethanol sensitivities depending on their subunit and splice variant composition (Chu et al., 1995; Simson et al., 1993; Woodward, 2000). Thus, NMDA receptors composed of either NR1/NR2A or NR1/NR2B subunits are more sensitive to ethanol's inhibitory effects than those composed of NR1/NR2C or NR1/NR2D subunits (Allgaier, 2002). Previous studies have shown that the NMDA receptor function is enhanced after prolonged forced ethanol treatment following withdrawal (Hu and Ticku, 1995; Iorio et al., 1992) and we could show in our animal model that long-term voluntary alcohol drinking leads to a significant increase in NMDA receptor function in the rat striatum (Darstein et al., 1998). We hypothesize that this might not only reflect an increase in NMDA receptor density, but that differential transcriptional regulation and alternative splicing of the various subunits comprising the NMDA receptor may lead to changes in receptor composition and subsequent function. We therefore examined the relative mRNA expression of NR1 splice variants in addition to the relative mRNA expression of NMDA receptor subunits NR2A, NR2B and NR2C (but not NR2D which is expressed at very low levels in the brain of adult animals) in the described rat model of long-term, voluntary alcohol self-administration.

2. Materials and methods

2.1. Animals

Nine male Wistar rats (Max Planck Institute of Biochemistry, Martinsried, Germany) weighing 220–250 g upon arrival in the laboratory, were used in this study. All animals were housed individually in standard hanging rodent cages with food and tap water *ad libitum*. Artificial light was provided daily from 7:00 a.m. to 7:00 p.m. and room temperature and humidity were kept constant (temperature: 23 ± 1 °C; humidity: $60 \pm 5\%$). The experiments were approved by the Committee on Animal Care and Use and carried out following the German Law on the Protection of Animals.

2.2. Long-term alcohol self-administration

After 1 week of habituation to the animal room, rats of the experimental group ($n = 5$) were given access to tap water, and alcohol solutions 5%, 10% and

20% (v/v) in their home cages. Alcohol solutions were made up from 96% pure ethanol diluted with tap water. Spillage and evaporation were minimized by the use of bottle caps with ball bearings (Ehret, Emmendingen, Germany). With this procedure the alcohol concentration in the solutions remained constant for at least 1 week. All drinking solutions were renewed weekly and positions of the four bottles were changed to avoid location preferences. Alcohol solutions were repeatedly withdrawn for 3 days (deprivation phase) every 4 weeks after the first 8 weeks of continuous access. The control group ($n = 4$), restricted to tap water, remained alcohol-naive. The average daily alcohol intake of these rats over the course of the experiment was approximately 6.5 g/kg. For further details regarding the long-term alcohol self-administration model please refer to the review by Spanagel and Holter (1999).

2.3. Experimental procedure

Following 2 years of voluntary alcohol drinking in the four-bottle model, described above, the alcohol drinking group was deprived of alcohol for 24 h. Their average intake during the week before sacrifice, which was after an alcohol deprivation phase, so demonstrating an alcohol deprivation effect, was 9.1 ± 0.48 g/kg.

Animals were then anaesthetized with halothane and decapitated. Brains were removed quickly, washed with ice-cold PBS and dissected on a plate cooled with ice. Brains were then divided into five brain regions: frontal cortex, occipital cortex, hippocampus, striatum (caudate and putamen) and cerebellum. Each tissue was wrapped individually and deep frozen on dry ice. All tissues were stored at -80 °C until further processing.

2.4. RNA-extraction

Tissue (25–30 mg) from each region and animal was homogenized initially using a syringe followed by a shredder column (Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. After elution in 40 μ l RNase-free water, concentration (app. 200 μ g/ml) and purity of the total RNA were determined by UV-spectroscopy with a Genequant photometer (Pharmacia Biotech). The RNA samples were stored at -80 °C until further processing.

2.5. RT-PCR

Total RNA was converted into cDNA using the Superscript^{TMII} Pre-amplification System (Life Technologies, Karlsruhe, Germany). The cDNA protocol included the following steps: 1 μ l oligo(dT) primer (0.5 μ g/ μ l) was added to 1.0 μ g mRNA and incubated at 70 °C for 10 min, followed by chilling on ice. Next, 4 μ l 5 \times First Strand Buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂) 2 μ l 0.1 M DTT (dithiothreitol), 4 μ l 10 mM dNTP Mix (2.5 mM of each dNTP) and 0.5 μ l RNase-inhibitor (40 units/ μ l) were added and the reactions incubated for 2 min at 42 °C. Following the incubation, 1 μ l RNase H⁻ Reverse Transcriptase (200 U/ μ l) was added and the sample incubated for a further 50 min at 42 °C. The reaction was terminated by incubation at 70 °C for 15 min. All cDNA samples were stored at -80 °C.

2.6. PCR

Intron-spanning primers were designed to allow detection of possible genomic DNA contamination. Cycle optimization was performed for each reaction to ensure that the detected PCR products represent the log-linear phase of amplification. PCR reactions were performed in a volume of 50 μ l containing 0.2 μ l (1 U) Taq-DNA Polymerase, 3 μ l (3 μ M) of the specific sense and anti-sense primer, 5 μ l (2.5 mM) of each dNTP, 10 μ l of specific 5 \times PCR buffer, 2 μ l cDNA and 26.8 μ l dH₂O (all manufactured by Life Technologies, Karlsruhe, Germany). All buffers contained 60 mM Tris-HCl and 15 mM ammonium sulfate with different magnesium concentrations: 2 mM, pH 9.0 for NR1 + E5/–E5; 1.54 mM, pH 9.0 for NR2A/2C; 1.5 mM, pH 8.5 for GAPDH/NR2B. All reactions were performed in a Thermocycler (Mastercycler Gradient[®], Eppendorf, Hamburg, Germany).

2.7. Exon 5 inclusion/exclusion isoforms of the NR1 receptor subunit

E5 sense 5'-TCA GCG ACG ACC ACG AGG GAC G and E5 antisense 5'-TTG TAG ATG CCC ACT TGC ACC A primers were used, as previously described (Allgaier et al., 1999). PCR reactions involved an initial 3 min denaturation at 94 °C followed by 33 cycles (94 °C 0:30 min, 64 °C 0:30 min and 2 °C 1 min). The amplified product lengths were 667 bp for the splice variant including exon 5 (NR1 + E5) and 604 bp for the product lacking exon 5 (NR1 – E5).

2.8. NR2A and NR2C subunits

Common primers detecting conserved regions of NR2A/2C were: sense 5'-GGG GTT CTG CAT CGA CAT CC-3' and antisense 5'-GAC AGC AAA GAA GGC CCA CAC-3'. The PCR reaction constituted an initial denaturation at 94 °C for 2 min followed by 33 cycles (94 °C 0:30 min, 53 °C 0:30 min and 72 °C 0:36 min). The amplified product, including both subunits NR2A and NR2C, had a size of 547 bp (Audinat et al., 1994) and was digested by restriction enzymes BpmI and ScaI (New England Biolabs, Frankfurt, Germany) which selectively cut the NR2A (5'...CTGGAG(N)16▼...3') and NR2C subunit (5'...AGT ▼ACT...3'), respectively. The digested fragments were of size of 226 bp and 321 bp for NR2A, and 186 bp and 361 bp for NR2C (Audinat et al., 1994).

2.9. NR2B subunit

To measure the relative mRNA expression of the NR2B subunit, the house-keeping gene glyceraldehyde dehydrogenase (GAPDH) was used as an internal standard. Both primer pairs were designed to anneal to conserved regions of rat sequences: NR2B sense 5'-GTG AGA GCT CCT TTG CCA AC-3' and NR2B antisense 5'-TGA AGC AAG CAC TGG TCA TC-3'; GAPDH sense 5'-TTC ACA AAG TTG TCA TTG AG-3' and GAPDH antisense 5'-TAC ATG GTC TAC ATG TTC CA-3'. The PCR reaction consisted of an initial denaturation at 94 °C for 2 min followed by 35 cycles (94 °C 0:30 min, 52 °C 0:36 min and 72 °C 2:00 min). The NR2B PCR product was 157 bp long and the GAPDH PCR product was 803 bp. The NR2B was not investigated in the striatum due to the limited amount of tissue available from this region.

2.10. Quantification of results

PCR products were resolved on a 2% agarose-gel (3% for the restriction digest) and stained with ethidium bromide. Electrophoresis was carried out at 100 V for 30–60 min. Bands were quantified directly from the gel using the Eagle Eye® system (Stratagene, Amsterdam, The Netherlands). Statistical analyses were performed using the SPSS 12.01 Software (Statistical Package for Social Sciences, SPSS Inc., Chicago, 2003). T-tests were used to evaluate possible differences in relative mRNA expression of NR1 exon 5 inclusion/exclusion; NR2C/NR2A, and GAPDH/NR2B. Analyses involved two-tailed estimations of significance, with an α -significance level of $p < 0.05$ defined to be statistically significant.

3. Results

Long-term voluntary alcohol self-administration shifted the ratio of NR1 exon 5 inclusion/exclusion towards exon 5 inclusion in the striatum of ethanol-treated rats compared to controls (Table 1; Fig. 1; d.f. = 7, $p = 0.02^*$), whereas no change was detectable in hippocampus, cerebellum, occipital and frontal cortex (Table 1).

Interestingly, the same region displayed also a change in the relative mRNA expression of NR2C/NR2A subunits. As shown in Fig. 2, mRNA expression of NR2A was increased when compared to NR2C subunits in the striatum (Table 2; Fig. 2; d.f. = 7, $p = 0.009^*$). No differences were detected in all other regions tested (Table 2).

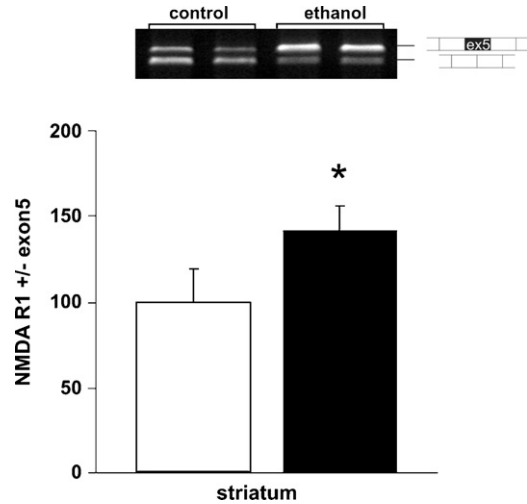


Fig. 1. Ratio of NR1 exon 5 isoforms in striatum following long-term alcohol self-administration. Results from RT-PCR analysis from two representative controls and ethanol-treated rats are shown on top. The structure of the gene is schematically indicated. Mean ratio values (\pm S.D.) between exon 5 inclusion and exon 5 skipping were calculated for controls ($n = 4$, white column) and ethanol-treated rats ($n = 5$, black column). Mean ratio for control tissue was arbitrarily set to 100%. The asterisk indicates significant differences ($p = 0.02$).

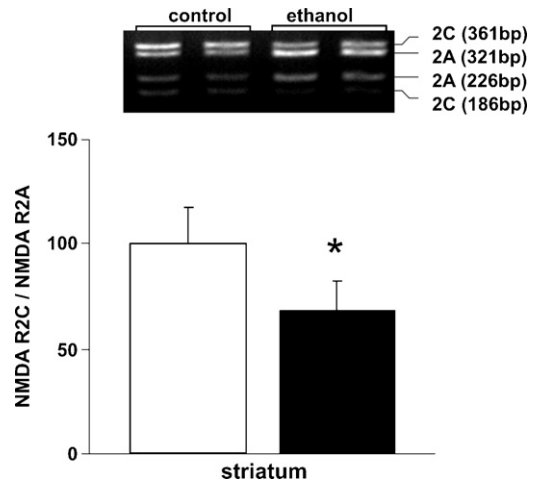


Fig. 2. Ratio of NR2C/NR2A mRNA in striatum following long-term alcohol self-administration. Results from RT-PCR analysis from two representative controls and ethanol-treated rats are shown on top. Restriction digest resulted in 226 bp/321 bp fragments (NR2A) and 186 bp/361 bp (NR2C). Mean values (\pm S.D.) of the relative NR2C/NR2A mRNA expression were calculated for controls ($n = 4$, white column) and ethanol-treated rats ($n = 5$, black column). Mean of ratio for control tissue was arbitrarily set to 100%. Asterisk indicates significant differences ($p = 0.009$).

We also observed a trend towards a relative increase of the NR2B subunit in the cerebellum of the same animals (Table 3; Fig. 3; d.f. = 7, $p = 0.098^+$). Experiments with frontal cortex tissue displayed no significant changes (Table 3).

4. Discussion

Taken together, our results demonstrate that long-term voluntary alcohol self-administration with repeated alcohol deprivation phases shifted the ratio of NR1 + exon 5/–exon 5

Table 1
Relative NMDAR1 + exon 5/–exon 5 mRNA expression

NR1 + E5/–E5	Group	N	Mean (S.D.)	T	d.f.	p-Value
Hippocampus	Ethanol	5	37 (6)	–0.059	7	0.954
	Control	4	38 (2)			
Striatum	Ethanol	5	126 (19)	3.008	7	0.02*
	Control	4	89 (18)			
Cerebellum	Ethanol	5	213 (13)	0.459	7	0.660
	Control	4	203 (44)			
Occipital cortex	Ethanol	5	31 (9)	0.123	7	0.906
	Control	4	30 (16)			
Frontal cortex	Ethanol	5	87 (26)	–1.503	7	0.177
	Control	4	115 (30)			

Table 2
Relative expression of NR2C/NR2A mRNA

NR2C/NR2A	Group	N	Mean (S.D.)	T	d.f.	p-Value
Hippocampus	Ethanol	5	99 (14)	–1.236	7	0.256
	Control	4	111 (15)			
Striatum	Ethanol	5	210 (30)	–3.58	7	0.009*
	Control	4	309 (53)			
Cerebellum	Ethanol	5	256 (66)	–1.399	7	0.205
	Control	4	305 (21)			
Occipital cortex	Ethanol	5	184 (49)	0.195	7	0.851
	Control	4	179 (30)			
Frontal cortex	Ethanol	5	287 (74)	0.898	7	0.399
	Control	4	251 (34)			

towards exon 5 inclusion, and of NR2A/NR2C ratio towards a relative NR2A increase in the striatum but not in any other region tested.

The used animal model based on voluntary long-term alcohol intake displays important features of alcohol addiction (Spanagel and Holter, 1999; Sanchis-Segura and Spanagel, 2006).

We observed regionally specific changes regarding the differential mRNA expression of NR1 splice variants and NR2 subunits when compared to models using forced alcohol intoxication. Follesa and Ticku (1995) observed no change of NR2A and NR2C mRNA levels in cerebellum, but elevated NR2A and NR2B mRNA subunits in cortex and hippocampus 9 h after the last ethanol administration which returned to control levels after 48 h. Hardy et al. (1999) observed an elevated NR2B mRNA expression which returned to control levels 18 h after

withdrawal, an altered NR1 ratio during ethanol exposure up to 48 h after withdrawal, and no change in NR2A and NR2C mRNA in the cortex. Furthermore, while Narita et al. (2000) observed no change in the cortex, the authors observed elevated NR2B protein levels in the limbic forebrain which gradually returned to baseline within 48 h after the withdrawal. The differences between the studies may reflect differences in the daily dose of alcohol consumed, the duration of alcohol consumption and the route of alcohol administration which are substantially different from those used in other studies. While we used a long-term (2 years), voluntary ethanol exposure (rats had the choice between water and ethanol in several concentrations), Follesa and Ticku (1995) administered ethanol by gavage. Animals were given 20% (v/v) ethanol, in saline, three times each day for 5 days and the doses were adjusted individually for each animal, according to the presence or absence of ataxia, loss of

Table 3
Relative expression of GAPDH/NR2B mRNA

GAPDH/NR2B	Groups	N	Means (S.D.)	T	d.f.	p-Value
Cerebellum	Ethanol	5	583 (170)	–1.906	7	0.098*
	Controls	4	992 (448)			
Frontal cortex	Ethanol	5	282 (44)	–0.756	7	0.474
	Controls	4	301 (22)			

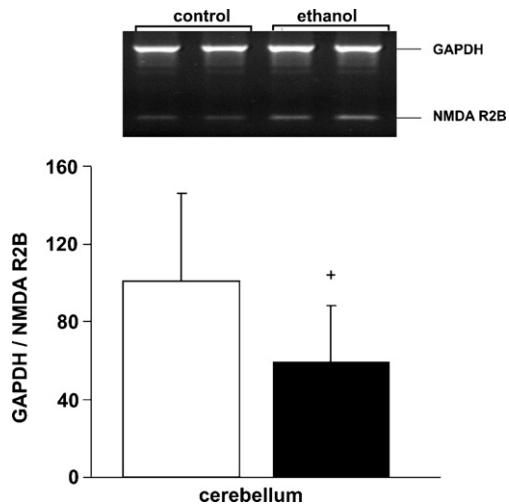


Fig. 3. (a) Ratio of GAPDH/NR2B in cerebellum following long-term alcohol self-administration. Results from RT-PCR analysis from two representative controls and ethanol-treated rats are shown on top. Mean values (\pm S.D.) of the relative GAPDH/NR2B mRNA expression were calculated for controls ($n=4$, white column) and ethanol-treated rats ($n=5$, black column). Mean of ratio for control tissue was arbitrarily set to 100%. Symbol “+” indicates a trend ($p=0.098$).

righting reflex, and motor activity. The ethanol-treated rats manifested episodes of severe intoxication. The rats used by Hardy et al. (1999) were exposed to ethanol vapor to maintain a blood ethanol concentration of 200–400 mg/dl for a period of 16 days. Finally, the mice used by Narita et al. (2000) were given access to a measured amount of liquid diet containing 5% (w/v) ethanol as their sole nutrient source for 5 days.

Ethanol accumulates in the brain with concentrations of ethanol being highest in the striatum (Chen et al., 2007). In our study, the changes in NMDA receptor composition were observed in the striatum but not in any other regions tested. Interestingly, Darstein et al. (1998) observed an increased NMDA receptor activity in the striatum in rats derived from our long-term drinking model. These findings suggest that the striatum may be particularly affected by long-term voluntary alcohol self-administration and withdrawal. Whereas the nucleus accumbens plays a critical role in the establishment of drug-seeking behavior (Spanagel and Weiss, 1999), the dorsal striatum progressively takes on a central role as drug-seeking becomes well established and may be more important in the maintenance and expression of an addictive-like behavior (Everitt et al., 2001; Everitt and Robbins, 2005; Vanderschuren et al., 2005, reviewed in Hyman et al., 2006). In fact, we could recently show that dopaminergic signaling in the striatum is more important in eliciting a relapse-like behavior (i.e. ADE) than the nucleus accumbens in rats deriving from our long-term voluntary alcohol self-administration procedure (Vengeliene et al., 2006).

The notion that not only the mere up-regulation but also the composition of NMDA receptors may play a crucial role in ethanol actions is supported by a recent study in which all eight NR1 splice variants were individually coexpressed with each NR2 subunit in human embryonic kidney 293 (HEK293) cells and tested for inhibition by ethanol using patch-clamp (Jin

and Woodward, 2006). All 32 subunit combinations tested gave reproducible glutamate-activated currents and all receptors were inhibited to some degree by 100 mM ethanol. Interestingly, in this study the sensitivity of individual receptors to ethanol was markedly affected by NR2 receptor composition and the specific NR1 splice variant expressed exhibiting up to 10-fold differences. At physiological pH, splice variants that include exon 5 are fully active, whereas those that lack exon 5 are partially blocked (Traynelis et al., 1995). It has been suggested that the exon 5 cassette forms a surface loop, with structural similarities to polyamines, and acts as a tethered modulator to shield the proton-sensor of NR1 (Traynelis et al., 1995). Isoforms containing exon 5 are therefore neither potentiated by polyamines nor inhibited by Zn^{2+} (Traynelis et al., 1998). It has also been shown that splicing of exon 5 can influence the deactivation properties of NMDARs (Rumbaugh et al., 2000). The deactivation time of recombinant NR2B-containing receptors is dependent on whether or not NR1 contains the exon 5 insert (Vicini et al., 1998). The deactivation rate is roughly four times faster for exon-5-containing NR1/NR2B receptors than for the exon-5-lacking NR1/NR2B receptors (reviewed in Cull-Candy et al., 2001). The NR1 + E5 splice variant is expressed mainly in parvalbumin-positive interneurons, which receive excitatory input from the cortex and inhibit medium-sized spiny striatal projection neurons (Tepper and Bolam, 2004), suggesting putative functional alterations in striatal information processing. Furthermore, an increased relative expression of NR2A compared to NR2C was detected in the striatum. This finding is in accordance with a higher expression of NR2A mRNA in GABAergic neurons after withdrawal from forced ethanol intake (Darstein et al., 2000). Parvalbumin-positive interneurons are one of the main neuronal populations expressing NR2A in the striatum.

In conclusion, our results demonstrate that long-term voluntary alcohol self-administration selectively affects the regulation of genes encoding the various subunits and splice variants of the NMDA receptor in the striatum.

Conflict of interest

All authors declare that they have no conflicts of interest.

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